UNIVERSIDADE DE LISBOA

FACULDADE DE FARMÁCIA

AKT2 siRNA-NANOPARTICULATE SYSTEM AS A NEW TOOL TO RESTORATION OF E-CADHERIN AND ERADICATE TUMOR METASTIC PHENOTYPE

DIANA FERNANDES DE SOUSA RAFAEL

Orientadores: Professora Doutora Mafalda Ascenção Marques Videira
Professora Doutora Helena Fialho Florindo
Professor Doutor Simó Schwartz Navarro

Tese especialmente elaborada para a obtenção do grau de Doutor em Farmácia, especialidade Tecnologia Farmacêutica

Júri

Presidente: Doutora Matilde da Luz dos Santos Duque da Fonseca e Castro, Professora Catedrática e Directora da Faculdade de Farmácia da Universidade de Lisboa.

Vogais:

- Doutor Luís Fernando Morgado Pereira Almeida, Professor Auxiliar da Faculdade de Farmácia da Universidade de Coimbra;
- Doutora Fernanda Raquel da Silva Andrade, *Research Scientist* do *Biomedical Research Networking Center in Bioengineering, Biomaterial and Nanomedicine*, Espanha;
- Doutora Luísa Maria Ferreira Romão Loison, Investigadora Principal do Instituto Nacional de Saúde Dr. Ricardo Jorge;
- Doutor Sérgio Jerónimo Rodrigues Dias, Professor Associado Convidado da Faculdade de Medicina da Universidade de Lisboa;
- Doutora Maria Beatriz da Silva Lima, Professora Catedrática da Faculdade de Farmácia da Universidade de Lisboa;
- Doutor Luís Filipe Batista Pleno de Gouveia, Professor Auxiliar da Faculdade de Farmácia da Universidade de Lisboa;
- Doutora Mafalda de Castro Ascensão Marques Videira, Professora Auxiliar da Faculdade de Farmácia da Universidade de Lisboa.

2017


FCT
Fundação para a Ciência e a Tecnologia
MINISTÉRIO DA EDUCAÇÃO E CIÊNCIA


LISBOA
UNIVERSIDADE
DE LISBOA


FACULDADE DE
FARMÁCIA
Universidade de Lisboa


iMed.
ULisboa
Research
Institute for
Medicines


Vall d'Hebron
Institut de Recerca


ciber-bbn
Biomedical Research Networking Center
Bioengineering, Biomaterials, Nanomedicine

Acknowledgements

Starting with the institutional acknowledgments, I would like to thank to Fundação para a Ciência e a Tecnologia (FCT) for financial support through the grant SFRH/BD/76270/2011 financed by the Programa Operacional Potencial Humano (POPH) do Quadro de Referência Estratégico Nacional (QREN) Portugal 2007-2013, and by funds from the Ministério da Ciência, Tecnologia e Ensino Superior (MCTES).

To Instituto de Investigação do Medicamento da Faculdade de Farmácia, Universidade de Lisboa (iMed.ULisboa) for had receive me as a PhD student.

To Faculdade de Farmácia da Univeridade de Lisboa, all the Professors and non-teaching staff of this institution that directly or indirectly make part of my life since 2005.

To my supervisor Professor Mafalda Videira, I am grateful for all the opportunities given in the last 6 years. They allow me to learn a lot, to have remarkable experiences, to travel around the world, to know incredible people, and to growth as a person.

A deep acknowledgment to Dr. Simó Schwartz Jr. for receive me at CIBBIM-Nanomedicine, for all the support, for the trust and for the wonderful opportunities. Truly, thank you very much for all the help.

To the innumerous colleagues and friends that I found during this long way at the different laboratories where I have been. Thank you for always receive me well and make the work easier and funniest.

To Petra and Fernanda because without them this thesis would never be possible. You know how much I will be always grateful for everything.

To my closest friends with whom I shared fantastic moments, support me in the not so good moments, and advised me when I must needed.

To my family, you are everything I am, my biggest support, my safe harbor and the reason of being who I am. My beloved parents and grandparents you will be always the most important thing in my life.

Abstract

Cancer has currently a major impact in the worldwide health, with breast and colon carcinomas presenting the second and third highest incidence rates, respectively. Despite the important advances in understanding the mechanism of carcinogenesis, disease progression, and development of new and more effective therapies, advanced cancer is still generally an incurable disease. The currently used treatments have improved both the survival and cure rates of patients; however there are some serious drawbacks, mostly concerning high toxicity and serious side-effects, which may cause even the death of a small percentage of patients. Furthermore, many patients suffer from tumors that are resistant to the conventional treatments, thus not taking profit from any therapeutic benefit. The resistance seen in the aggressive forms of the disease remains to be the biggest challenge of current treatments. More than half of the treated patients suffer from a disease relapse, most of them with distant metastases. Cancer maintenance, resistance to therapy, and metastatic disease seem to be sustained by the presence of cancer stem cells (CSC) within the tumors. These cells retain the capacity of repopulating the tumor after the treatment, while being insensitive to conventional anticancer therapies, antimitotic agents or radiation. It is still not entirely clear if CSC originate from the epithelial stem cells which are essential to maintain proliferative homeostasis or result by accumulation of mutations from differentiated cells. However it is known that only a few number of CSC are necessary and sufficient for tumor regeneration. In this scenario, new cancer therapies under development should fulfil two conditions: (i) reduce the toxicity displayed in current therapies and most importantly, (ii) specifically target and eliminate CSC to overcome drug resistance and tumor recurrence.

Both criteria could be accomplished using innovative targeted nanomedicines for drug and/or gene delivery, which is widely expected to bring promise and create novel therapeutics in advanced cancer therapy. Nanoparticles have shown, in both preclinical and clinical practice, to improve the therapeutic window of the therapeutic agents by specifically delivering higher concentrations to the tumor lesions, while reducing the systemic toxicity. This behavior explains the increased interest of research groups and pharmaceutical companies in nanomedicine-based systems to treat cancer, resulting in an increase in the approved and clinically tested products in the last decades.

The advances observed in molecular biology and biotechnology have been contributing for the identification and understanding of the complex biological pathways associated with cancer development, and consequently for the emergence of gene therapy as a promising

therapeutic alternative in diseases with a strong genetic component. New biomarkers and therapeutic targets have been identified. AKT2 is one of the three Protein (serine/threonine) Kinase B (PKB or AKT) isoforms that is amplified in solid tumors. This oncogene is increased in response to apoptosis and its activation is also associated with phosphoinositide 3-kinase (PI3K) related effects as well as TWIST-promoted metastatic process by enhancing cell migration and invasion. Activation of TWIST/AKT2 signaling pathway is involved in E-cadherin silencing in the population of CSC, conferring them high tumorigenic potential. Thus, a therapeutic RNAi-mediated strategy using AKT2 as a downstream target represents an enormous step forward CSC eradication and anti-cancer therapy.

The effects of AKT2 silencing in different breast and colon cancer cells, expressing and non-expressing TWIST are discussed in the present work. The obtained results demonstrated that the AKT2 inhibition is effective in terms of reducing cells migration, invasion, and transformation via different mechanisms and do not depend on the presence of TWIST within the tumor. We were able to observe that for cell lines expressing TWIST, AKT2 acts via Epithelial-Mesenchymal Transition (EMT) reversion, while for cells not expressing TWIST, AKT2 acts via mTOR pathway promoting the reduction of stemness markers and alteration in the expression of apoptotic genes such as the Bcl-2 and p53. In summary, the aim of this work was to silence the AKT2 expression using an small interfering RNA (siRNA) against the AKT2 (siAKT2) in different cancer cells lines in order to prove that, not only in bulk cancer cells but also in the sub-population of CSC, the AKT2 knockdown is able to impair their increased tumorigenic ability, reverting their mesenchymal phenotype, reducing cell invasion, inhibiting colony formation or inducing apoptosis. Due to the effect of AKT2 in both bulk cells and CSC, the therapy with siAKT2 could be used as two different strategies: i) to revert the EMT and maintain the tumors in its primary stage for easier chirurgical removal, and ii) prevent the tumor recurrence through the inhibition of CSC tumorigenic and metastatic potential.

Despite the enormous outbreak of gene therapy in the last years, its clinical use is still limited mainly due to genetic material-associated delivery difficulties, such as fast degradation, insufficient transfection efficiency or dose limiting vectors toxicity. In order to overcome these problems, we pursued the development and validation of a nanocarrier composed by Pluronic® F127 micelles associated with polyethylenimine (PEI)-based polyplexes to deliver a siAKT2. The proposed system for the delivery of siAKT2 seems to gather the requirements for an efficient and safe transport of siRNA in terms of their physicochemical characteristics, internalization capacity, biological efficacy and toxicity profile. These results make us believe that this new formulation will constitute a

technological platform for the development of systems to encapsulate different siRNAs, as well as other types of genetic material. We are putting efforts in the improvement of these formulation in order to achieve a potential gene or multifunctional delivery system, approaching the so increasingly required personalized and combined therapies and bringing new hope into the field of cancer therapy.

Keywords: Breast and Colon Cancer, AKT2, TWIST, Epithelial-Mesenchymal Transition, mTOR pathway, Cancer Stem Cells, Gene Delivery, siRNA, Nanoparticles, Polymeric Micelles.

Resumo

As doenças oncológicas possuem um enorme impacto na saúde das populações a nível mundial, apresentando o cancro da mama e o do cólon a segunda e terceira maior taxa de incidência, respetivamente. Apesar dos importantes avanços observados nos últimos anos na compreensão dos mecanismos de carcinogénese e progressão da doença, bem como no desenvolvimento de terapias mais eficazes, o cancro em estadios mais avançados é ainda geralmente uma doença incurável. Os tratamentos usados atualmente têm vindo a melhorar as taxas de sobrevivência e de cura dos doentes, contudo existem ainda algumas dificuldades especialmente relativas à elevada toxicidade e aos efeitos secundários severos, responsáveis até pela morte de uma pequena percentagem dos doentes. Adicionalmente, muitos doentes apresentam tumores que são resistentes aos tratamentos convencionais, não alcançando assim nenhum benefício terapêutico. A resistência à terapêutica observada nas formas mais agressivas da doença continua a ser o maior desafio dos tratamentos atuais. Mais de metade dos doentes tratados com terapias convencionais sofrem de uma recidiva da doença, na sua maioria com metástases distantes. A manutenção do cancro, a resistência à terapia e o potencial metastático da doença parece ser sustentado pela presença de células estaminais cancerígenas (CEC) no microambiente do tumor. Estas células retêm a capacidade de repopular o tumor após o tratamento e são insensíveis aos tratamentos convencionais com compostos antimitóticos ou à radiação.

Ainda não está absolutamente esclarecido se as CEC são originadas a partir das células estaminais epiteliais, essenciais à manutenção da proliferação homeostática, ou se resultam da acumulação de mutações de células diferenciadas. No entanto, estudos demonstram que apenas um pequeno número de CEC é suficiente para provocar a regeneração do tumor. Perante este cenário, as novas terapêuticas para o cancro em desenvolvimento devem satisfazer duas condições: 1) reduzir a toxicidade causada pelas terapias atuais e, mais importante ainda, 2) atingir e eliminar especificamente as CEC de forma a evitar a resistência à terapêutica e a recorrência do tumor. Ambas as condições podem ser alcançadas usando terapêuticas inovadoras baseadas em nanomedicina direcionada para a veiculação de fármacos e/ou material genético, nas quais se deposita uma nova esperança no campo da terapia contra o cancro avançado. As nanopartículas têm demonstrado, tanto na prática clínica como na pré-clínica, serem capazes de melhorar a janela terapêutica de um determinado fármaco ao permitir a entrega de maiores

concentrações do mesmo às lesões tumorais, reduzindo ao mesmo tempo a sua toxicidade sistémica. Este comportamento explica o interesse crescente que se tem verificado por parte de diversos grupos de investigação e indústrias farmacêuticas na nanomedicina para o tratamento do cancro. Consequentemente, nos últimos anos, tem-se verificado também um aumento na aprovação e entrada no mercado, bem como a aplicação clínica de produtos baseados em nanopartículas.

Os avanços na biologia molecular e biotecnologia têm contribuído para a identificação e compreensão das complexas vias biológicas associadas ao desenvolvimento do cancro e, consequentemente têm contribuído para o crescimento da terapia génica como uma alternativa terapêutica promissora para o tratamento de doenças com um forte componente genético. Novos biomarcadores e agentes terapêuticos têm sido identificados nos últimos anos.

O AKT2 é uma das três isoformas da proteína quinase B (PKB ou AKT) que está amplificada em tumores sólidos. Este oncogene está aumentado em resposta à apoptose e a sua ativação está também associada com os efeitos da fosfoinositídeo 3-quinase (PI3K) e com o processo metastático promovido pelo TWIST, nomeadamente o aumento da migração e invasão celular. A activação da via biológica TWIST/AKT2 está envolvida no silenciamento da E-caderina na população de CEC, o que lhes confere um elevado potencial tumorogénico. Desta forma, uma estratégia terapêutica baseada na tecnologia de RNA de interferência, usando a AKT2 como alvo, surge como uma alternativa importante na terapia oncológica e, mais importante, na erradicação das CEC. Os efeitos do silenciamento do AKT2 em diferentes linhas celulares de cancro da mama e do cólon, com e sem expressão de TWIST, são discutidos no presente trabalho. Os resultados obtidos demonstram que a inibição do AKT2 é eficaz na redução da migração, invasão e transformação celular via diferentes mecanismos e que não depende da presença de TWIST no tumor. Foi possível observar que para as linhas celulares que expressam TWIST, o AKT2 atua via redução da transição epitelial-mesenquimal (EMT), enquanto que em células que não expressam TWIST, o AKT2 atua pela via do mTOR promovendo a redução de marcadores de malignidade e alteração de genes apoptóticos como o Bcl-2 e o p53. Em resumo, o objetivo deste trabalho consiste no silenciamento da expressão do AKT2 usando pequenos fragmentos de RNA de interferência (siRNA) contra o AKT2 (siAKT2) com o objetivo de provar que, não apenas em linhas de cancro parentais mas também nas sub-populações de CEC, o silenciamento do AKT2 é capaz de comprometer as suas características tumorigénicas através da reversão do seu fenótipo mesenquimal, redução da invasão celular, inibição da formação de colónias ou indução da apoptose. Devido ao efeito observado do AKT2 nas células de cancro parentais e também nas CEC,

a terapia com siAKT2 poderia ser usada em duas possíveis estratégias: 1) para reverter o EMT e manter os tumores no seu estadio inicial, e como tal mais facilmente removíveis cirurgicamente, e ii) prevenir a recorrência do tumor através da inibição do potencial tumorigénico e metastático das CEC.

Apesar da enorme eclosão da terapia génica nos últimos anos, o seu uso clínico é ainda bastante limitado, maioritariamente devido às dificuldades relacionadas com a veiculação do material genético tais como a rápida degradação dos oligonucleótidos, a insuficiente eficiência de transfeção ou a toxicidade associada aos vetores usualmente utilizados. Com o objetivo de ultrapassar esses problemas, este trabalho teve como segundo objetivo o desenvolvimento e validação de uma formulação para a veiculação de siAKT2, consistindo em micelas de Pluronic® F127 associadas com poliplexos de polietilenoimina (PEI). O nanosistema proposto parece preencher os requisitos necessários a um transportador de siRNA seguro e eficiente em termos das suas características físico-químicas, capacidade de sofrer internalização pelas células, eficácia biológica, e perfil de toxicidade. Estes resultados promissores fazem-nos acreditar que esta nova formulação pode servir como uma plataforma tecnológica ao desenvolvimento de sistemas para encapsular diferentes siRNAs, bem como outros tipos de material genético. Reunimos esforços no sentido de melhorar esta formulação com o objetivo de alcançar um sistema de veiculação genético e/ou multifuncional promissor. Desta forma, será possível alcançar mais facilmente a desejada terapia combinada e personalizada e com o objetivo final de trazer uma nova esperança no campo da terapia contra o cancro.

Palavras-chave: Cancro da Mama e Colon, AKT2, TWIST, Transição Epitelial-Mesenquimal, via do mTOR, Células Estaminais Cancerígenas, Veiculação de Genes, siRNA, Nanopartículas, Micelas Poliméricas.

Table of Contents

List of Figures

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

List of Tables

Abbreviations

3'UTR	Untranslated region
5-DTAF	5-8[4,6-dichlorotriazin-2-yl]amino)fluorescein hydrocloride
ABC	ATP-binding cassette
AE	Association efficiency
AGO2	Argonaute 2
AKT	Protein (serine/threorine) kinase B (PKB)
ALDH1A1	Aldehyde dehydrogenase 1
Alox5	Arachidone 5-lipoxigenase
ATCC	American type cell colection
ATP	Adenosine triphosphate
BAD	Bcl2-associated death promoter
BCA	Bicinchoninic acid
Bcl-2	B-cell lymphoma 2
Bcl-xL	B-cell lymphoma extra large
BCRP	Breast cancer resistance protein
BH3	Bcl-2 Homology 3 domain
bHLH	basic helix-loop-helix
BLMH	Bleomycin hydrolase
CAMs	Cell adhesion molecules
CD	Cyclodextrin
CHEMS	Cholesteryl hemisuccinate
CMC	Critical miccelar concentration
CML	Chronic myeloid leukemia
CMT	Critical micellar temperature
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CryoSEM	Cyro-scanning electron microscopy
CS	Chitosan
CSC	Cancer stem cells
CTAB	Cetyltrimethylammonium bromide
CTLA4	CytotoxicT-lymphocyte antigen 4
CXCR4	C-X-C chemokine receptor type 4
DAPI	4',6-diamidino-2-phenylindole

DC	Denditric cells
DD	Degree of deacetylation
DDS	Drug delivery system
DLL	Delta-like ligand
DLS	Dynamic light scattering
DM	Dissolution method
DMEM	Dulbecco's modified Eagle medium
DMRIE	2,3-di(tetradecoxy)proply-2hydroxyethyl)-dimethylazanium bromide
DMSO	dimethyl sulfoxide
DOPC	1,2-dioleoyl-sn-glycero-3-phosphocholine
DOPE	1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphoethanolamine
DOPG	1,2-Dioleoyl-sn-glycero-3-phosphoglycerol
DOSPA	2,3dioleyloxy-N-[2(sperminecarboxamido ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate
DOSPER	1,3-dioleoyloxy-2-. (6-carboxyspermyl)-propyl amide
DOTAP	1,2-dioleoyl-3-trimethylammonium-propane
DOTMA	1,2-di-O-octadecenyl-3-trimethylammonium propane
DSPC	1,2-distearoyl-sn-glycero-3phosphocholine
dsRNA	Double-stranded RNA
DTA	Diphteria toxin
ECM	Extracellular matrix
EGFR	Epidermal grownth factor recptor
EMT	Epithelial-mesenchymal transition
EO	Ethylene oxide
EPR	Enhanced permeability and retention effect
ERK	Extracellular signal–regulated kinases
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FDA	Food and drug administration
FGFR	Fibroblast growth factor receptors
FH	Thin-film hydration
FR	Folate Receptor
GADPH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green fluorescent protein
GFP-siRNA	siRNA against GFP

GSKβ3	Glycogen synthase kinase 3 beta
HER2	Hormone Estrogen Receptor 2
HES	Hydroxyethyl starch
HIF-2α	Hypoxia-inducible factor 2 alpha
HLB	Hydrophilic lipophilic balances
HPMA	N-(2-hydroxypropyl)methacrylamide
IC_{50}	Inhibitory concentration 50
IFN-y	Interferon Y
IGF-1	Insulin-like growth factor 1
IGFR	Insulin-like growth factor receptor
IL-3	Interleukin-3
IL-6	Interleukin-6
ILK	Integrin-linked kinase
LinOS	N4-linoneolyl-N9-oleoyl-1,12-diamino-4,9-diazadodecane
Lipoplexes	Lipid-based Delivery Systems
LPP	Lipoplexes
MAPK	Mitogen-activated protein kinase
Md	Mean diameter
MDM2	Mouse double minute 2 homolog
MDR	Multidrug resistance
MDT	Maximum tolerated dose
MEK	Methyl ethyl ketone
MET	Mesenchymal-epithelial transition
miRNA	MicroRNA
MRD	Minimal residual disease
MRP	Multidrug resistance-associated proteins
MTD	Maximum tolerated dosis
mTOR	Mechanistic target of rapamycin
m-TORC1/2	Mammalian target of rapamycin complex 1 and 2
MTT	3-(4,5-dimethythiazol-2-yl)-2,5 diphenyl tetrazolium bromide
MW	Molecular weight
N/P ratio	Nitrogen/Phosphate ratio
NF-kB	Nuclear factor kappa B
Nio-AU	Gold niosomes
NK	Natural killer
OCT4	Octamer-binding transcription factor

OGN	Phosphate groups
PAEs	Poly(b-amino ester)s
PAMAM	Polyamidoamine
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline
PCL	Poly(ε-caprolactone)
PCSK9	Proprotein convertase subtilisin/kexin type 9
Pdi	Poydispersity index
PDK1	Phosphoinositide-dependent protein kinase 1
PDMAEMA	Poly(2-(N,N-dimethylamino)ethyl methacrylate**)**
PE	Phosphoethanolamine
PEG/PEO	Polyethylene glycol/ Polyethyleneoxide
PEI	Polyethylenimine
PCL	Polycaprolactone
PFA	Paraforlmaldehyde
PGE2	Prostaglandin E2
P-gp	P-glycoprotein
PH	Pleckstrin homology domain
PHML-b-PLLA-b-PHML	Poly(hydroxyletheyl methacrylate-L-lysine)-b-poly)L-lactide)—poly(hydroxyletheyl methacrylate-L-lysine
PI3K	Phosphoinositide 3-kinase
PILP	Pegylated immuno-lipopolyplexes
PKB	Protein kinase B
PLA	Poly(d,l-lactide)
PLAS	PEGylated lipoplex-entrapped alginate scaffold
PLGA	Poly(d,l-lactide-co-glycolide)
PLK1	Polo-like kinase
PLL	Poly(L-lysine)
PM	Polymeric micelles
PO	Propylene oxide
Poliplexes	Polymer-based Delivery Systems
Poll II	Polymerase II
PPEEA	Poly(2-aminoethyl ethylene phosphate)
PPO	Polypropylene oxice
PTEN	Phosphatase and tensin homolog
PTX	Paclitaxel

qRT-PCR	Quantitative real time polymerase chain reaction
RFP	Red Fluorescent Protein
RISC	RNA-induced silencing complex
RRM2	Ribonucleoside-diphosphate reductase subunit M2
RTK	Receptor tirosine kinase
SCID	Severe combined immunodeficiency
SD	Mean±standard desviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser	Serine
shRNA	Short hairpin RNA
SLN	Solid lipid nanoparticules
SLUG	Zinc finger protein SNAIL2
SNAIL	Zinc finger protein SNAIL1
SNALP	Stable nucleic acid lipid particles
SPARC	Secreted protein acidic and rich in cysteine
STAT3	Signal transducer and actibvator of transcription 3
SV40	Simian vacuolating virus 40
TBE	Tris/Borate/EDTA
TEM	Transmission electron microscopy
Tf	Human transferrin
TfR	Transferrin receptors
TGF-β	Tumor growth factor beta
TGFβR	Tumor growth factor beta receptor
Thr	Threonine
TNF	Tumor necrosis factor
TORC2	Target of rapamycin complex 2
TQ	Thymoquinone
UV	Ultra violet
VEGF	Vascular endothelial growth factor
WB	Western Blotting
WNT	Wingless-related integration site
ZEB	Zinc finger E-box-binding homeobox
ZP	Zeta potencial

Aims and Organization of the Thesis

The high complexity of cancer diseases and their still unknown and incontrollable pattern, has made prioritary the study and a better understanding of the wide range of biological pathways involved in their development and progress. Worstly, the heterogeneity of cancer cell populations within the tumor difficults the complete remission of the disease, being the cancer recurrence one of the major challenges for the current therapies. The small subpopulation of CSC within a certain tumor have been reported as the responsible for the so feared tumor recurrence and resistance to therapy.

This work could be divided in two main research areas and general objectives:

i) Molecular biology of cancer: study and identification of AKT2 as a good target for cancer therapy;

ii) Pharmaceutical technology and development: design and characterization of a Nanotechnology-based system for delivery of a siRNA against the AKT2.

Based on the previous, the specific objectives of this work are the follows:

1) Due to its recognized role as a putative oncogene, we aim to understand and characterize the biological pathway of AKT2 and assess the effects of AKT2 silencing in different breast and colon cancer cell lines in terms of cell migration, proliferation, and gene expression pattern;
2) Isolate the subpopulations of CSC from breast and colon cancer cells and assess the effects of AKT2 silencing specifically in this subpolation in terms of cell invasion, cell anchorage-independent growth, and gene expression pattern;
3) Understand the downstreams effectors of AKT2 for each cell line (TWIST or mTOR);
4) Design a nanoparticle-based system able to complex siRNA and silence the gene of interest using classic reporter gene assays;
5) Characterize the new nanosystem in terms of their physicochemical features and stability, *in vitro* and *in vivo* toxicity, and cellular internalization;
6) Assess the biological effects of nanoparticles complexing siRNA against the AKT2 in subpopulations of both breast CSC and non-CSC in terms of invasion and cell anchorage-independent growth abilities.

The present thesis is organized in six chapters. In the first two chapters are presented the main theoretical concepts and the state of art for each of research areas:

Chapter 1 – State of Art – Epithelial-Mesenchymal Transition as the Motor for Stemness: AKT2 Emerges as a Potential Therapeutic Target

Chapter 2 – State of Art – Nanotechnology for Gene Delivery

After the introductory section is presented the research work developed and the main results obtained:

Chapter 3 (Formulation Studies and Efficacy Assessment of Different siRNA Delivery Systems) describes the way paved through the design and development of a nanoparticulate system based in polymeric micelles (PM) for siRNA delivery until reach the final selected formulation presented in Chapter 5;

Chapter 4 (AKT2-related Biological Pathway Characterization and Validation in Breast and Colon Cancer Stem Cells) presents the study and characterization of the biological pathway and the role of AKT2 in the development and progression of cancer both in breast and colon CSC and non-CSC;

Chapter 5 (Functional Validation of Amphiphilic-based Polymeric Micelles for siRNA Delivery and Cancer Stem Cells Genes Inhibition) presents the results obtained with the chosen PM for the delivery of siAKT2.

In the final chapter (**Chapter 6**) are presented the Main Conclusions and Future Perspectives of the work.

CHAPTER 1

State of Art – Epithelial-Mesenchymal Transition as the Motor for Stemness: AKT2 Emerges as a Potential Therapeutic Target

The information presented in this chapter was partially published in the following publications:

1) P Gener, D Rafael, Y Fernández, J Sayos, D Arango, I Abasolo, M Videira, S Schwartz Jr., Cancer Stem Cells and Personalized Cancer Nanomedicine. Nanomedicine (Lond), 11(3):307-20, 2016.

2) D Rafael, S Doktorovová, H Florindo, P Gener, I Abasolo, S Schwartz Jr., M Videira, EMT Blockage Strategies: Targeting Akt Dependent Mechanisms for Breast Cancer Metastatic Behaviour Modulation, Current Gene Therapy, 15(3) 2015.

Table of contents

1.1. Cancer Facts

According to World Health Organization (WHO), cancer is a generic term used to describe a large and heterogeneous group of diseases that can affect any part of the body. A key feature is the genesis of abnormal cells that rapidly grow beyond their normal boundaries invading adjacent parts of the body and spreading to other organs (metastasis) **(Figure 1.1)**. Metastization is of major impact in the malignancy of the disease and clinical outcome (1). Due to its high diversity, complexity, and unpredictable and uncontrollable character, cancer has become one of the most feared diseases by humans in the last decades. With the arise of molecular biology and biotechnology, tremendous advances have been observed in the last decades in the oncology field leading to the identification of biological pathways involved in cell growth and dissemination, as well as disease players and therapeutic targets/agents **(Figure 1.1)**.

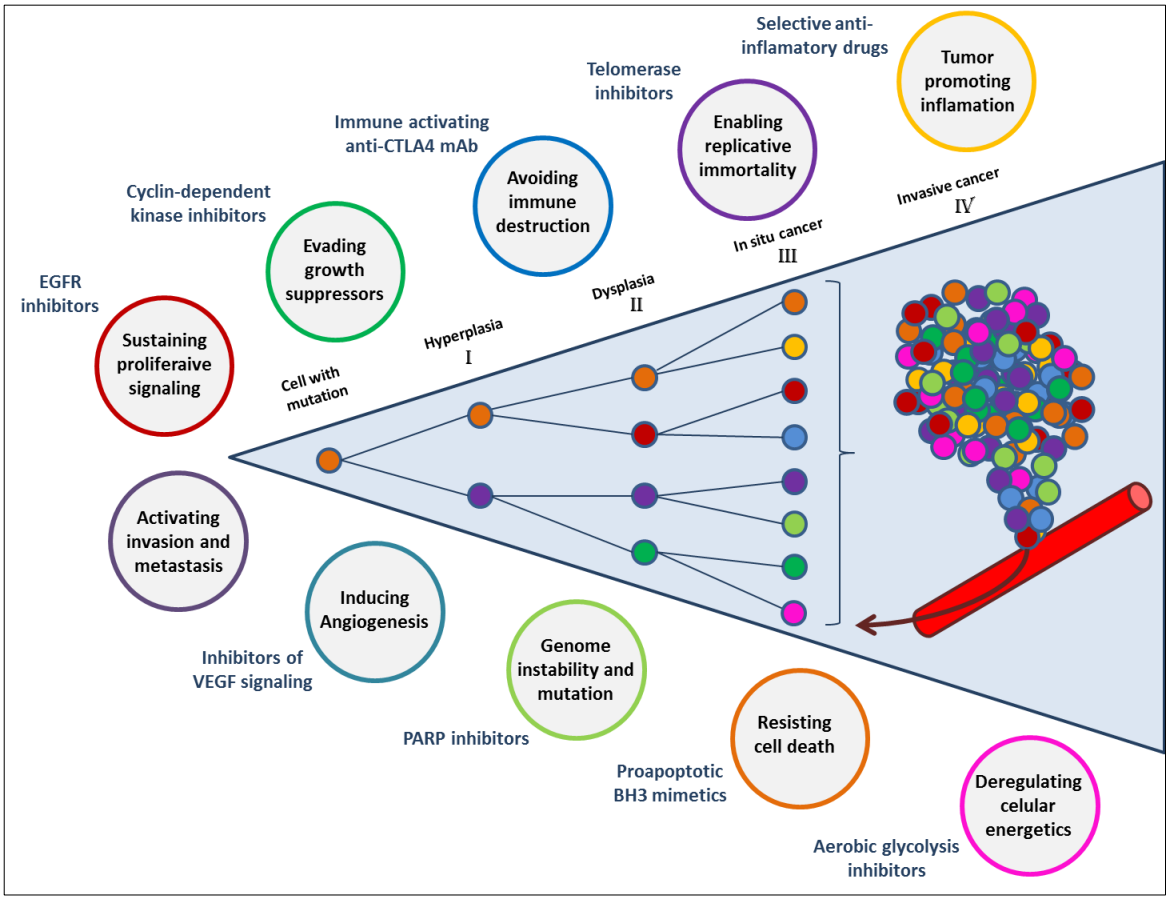


Figure 1.1. The complexity of cancer. The figure represents different phases of the disease evolution and the problematic of cancer mutations that increase exponentially along with the tumor development originating a highly heterogeneous compartment. Circles point out the main cancer hallmarks as well as the associated most common therapeutic strategies. BH3: Bcl-2 homology 3 domain; CTLA4: cytotoxic T-lymphocyte antigen 4; EGFR: epidermal growth factor receptor; PARP: poly (ADP-ribose) polymerase; VEGF: Vascular endothelial growth factor.

1.1.1. Cancer Scenario

The progresses referred before enables researchers and pharmaceutical industry with the information necessary to develop a variety of drugs to treat the different types of cancer, resulting in an important decrease of the cancer related deaths (superior to 20% since 1990s) and an increase in the patient's overall survival (2, 3). The incidence and mortality rates of this disease are superior in developed countries and are expected to increase in the next years, with an estimated 9 million cancer deaths in 2015 and 11.4 million in 2030 (3). Just in the USA, 1.7 million new cancer cases and 0.6 million cancer deaths are projected to occur in 2017 (4). Nowadays, the seven most common types of cancers worldwide are lung cancer, breast cancer, colorectal cancer, prostate cancer, stomach cancer, liver cancer, and cervical cancer **(Figure 1.2)** (5). Cancers typically associated with infections such as the cervical cancer, stomach cancer, liver cancer and Kaposi's sarcoma are declining in the developing countries as infections become better controlled with the programs implemented by WHO. On the contrary, lung, breast, prostate, and colorectal cancers present a faster rate of incidence, being the world cancer incidence predicted to rise 75% by 2030, mainly associated with the increasing bad lifestyle practices, population growth and aging (3, 5).

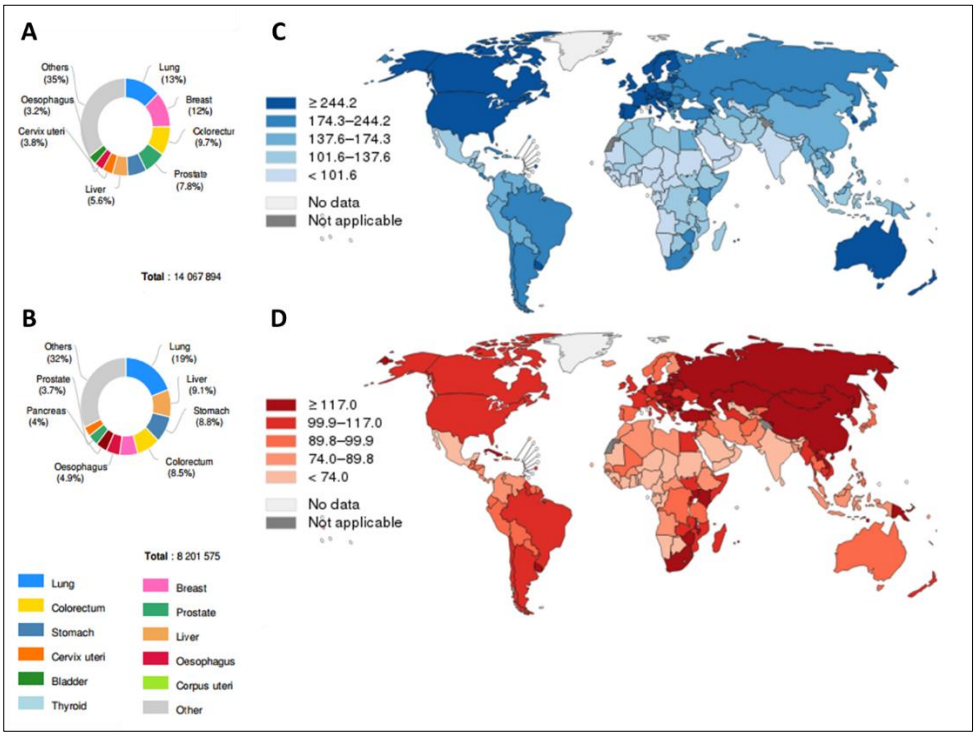


Figure 1. 2. Cancer statistics. Estimated incidence (A) and mortality (B) worldwide of the most common types of cancer in both sexes. Estimated age-standardized rates of incident cases (C) and deaths (D) in both sexes for all cancers excluding non-melanoma skin cancer. Data Source: GLOBOCAN 2012. Map Production IARC (2016) (5).

In the last years, due to the growing incidence of the disease, the global market share of medicines to treat cancer has been growing. In 2014 oncology remained as the largest therapeutic area worldwide regarding the market share, presenting an increase of 8% compared to 2013 and counting for more than 10% of the total year sales (6). As a consequence of the expected growth in the prevalence and incidence of oncological diseases in the upcoming years, it is also predicted an increase in the prescription of anticancer drugs, with an escalation of their global market share up to almost 15% in 2020 (6). This disease profile entails high costs to the health care systems related to the patients' cancer treatment and care. For example, only in USA, cancer care is projected to cost around 174 billion US dollars in 2020 (7). This scenario boosts researchers, health care agencies, and pharmaceutical companies to pursuit the urgent need for the development of new and more cost-effective treatments.

1.1.2. Cancer Treatment

Despite the enormous efforts employed at different fields of science and medicine, the real cure and eradication of cancer remains a desire far to be achieved and stands as a huge challenge to researchers around the world.

Major challenges surrounding the administration of current anticancer treatments are their systemic toxicity, associated with serious side-effects owing this drugs lack of specificity, and the therapeutic resistance that cancer cells often acquire during treatment. Indeed, because of the use of chemotherapeutics at an early stage of the disease that renders tumor resistance, treatment options for late metastatic disease are often reduced (8). The ability of cancer cells to activate alternative molecular pathways allowing them to escape from the common drug therapeutic mechanism of action is one of the major mechanisms involved in the development of drug resistance (9, 10). Further, drug resistance also occurs through the increased expression of ATP-dependent drug efflux transporters on the surface of cancer cells (11). This causes a significant decrease of intracellular drug accumulation, which results into severe limitation of drug's efficacy (12). Besides, cells with different phenotypes and proliferative abilities co-exist within a tumor, generating tumor heterogeneity and contributing to specific selection of resistant clones and disappointing therapeutic responses, in particular when a single drug treatment is applied.

Special interest has been paid to the use of biopharmaceuticals and gene therapy as therapeutic strategies to treat cancer owing to its capacity to target the specific antigen or signaling pathway involved in cancer progression (13). Among the different types of gene therapy, the silencing of particular genes through the RNA interference (RNAi) therapy has

been intensively investigated as a encouraging strategy in the field of oncology, given the possibility of targeting oncogenes involved in proliferation, survival, angiogenesis, metastasis, apoptosis suppression or drug-resistance (14).

Nowadays, more than 800 medicines and vaccines to treat cancer are under development and clinical assessment (2), with more than 73% of the medicines in the pipeline being studied based on biomarkers, presenting potential for personalized treatment (15). Some of the medicines under development are based on biopharmaceuticals, namely monoclonal antibodies, vaccines, cell or gene therapy. In 2013, the majority of biopharmaceuticals under development are intended to treat cancer and related diseases (more than 300), being the monoclonal antibodies the most studied ones with 170 medicines (16). Also, seventeen products under development/clinical trials were based on gene therapy such as ALN-VSP from Alnylam Pharmaceuticals (Phase I), B7-2/GM-CSF from NuVax Therapeutics (Phase I), BC-819 from BioCancell Therapeutics (Phase II), CALAA-01 from Calando Pharmaceuticals (Phase I), EGEN-001 from EGEN (Phase II) or GliAtak™ from Advantagene (Phase II) (16).

Unfortunately, even though important clinical breakthroughs in the fight against cancer have been achieved by using combined protocols such as conventional chemotherapy with hormonal therapy or therapeutic antibodies, recurrence and metastasis are still observed. Thus, the development of treatments able to reduce the formation of metastasis and to avoid or at least reduce the recurrence of the disease is required.

1.2. The Cancer Stem Cells Theory

As referred previously, despite progresses in cancer management, advanced cancers have still low clinical response rates and high recurrence and mortality rates. Regarding this, a main issue that should be considered is the fact that cancer cells within a tumor are not equal in terms of characteristics and tumorigenic potential.

1.2.1. Cancer Stem Cell Models

Before the 1990s, cancer initiation and progression was explained by a clonal cancer model. It was considered that all tumor cells have similar characteristics and equal tumor formation capacity and that tumors expansion depended on clonal selection advantages (17-20). The first evidence regarding the existence of cancer stem cells (CSC) was obtained in acute myeloid leukemia (21). Subsequently, CSC were identified also in other hematopoietic

cancers and in many solid tumors (breast, brain, colon, prostate, lung, head & neck, among others) (22-24). It was also shown that the small CSC sub-population has the ability to generate and maintain the tumor progression (20-22, 25). Based on these observations, a new hierarchical model describing cancer propagation was postulated (21); CSC can self-renew their own population and have long-term propagating capacity contrariwise to normal cells whose division finish by clonal exhaustion **(Figure 1.3A)** (26, 27).

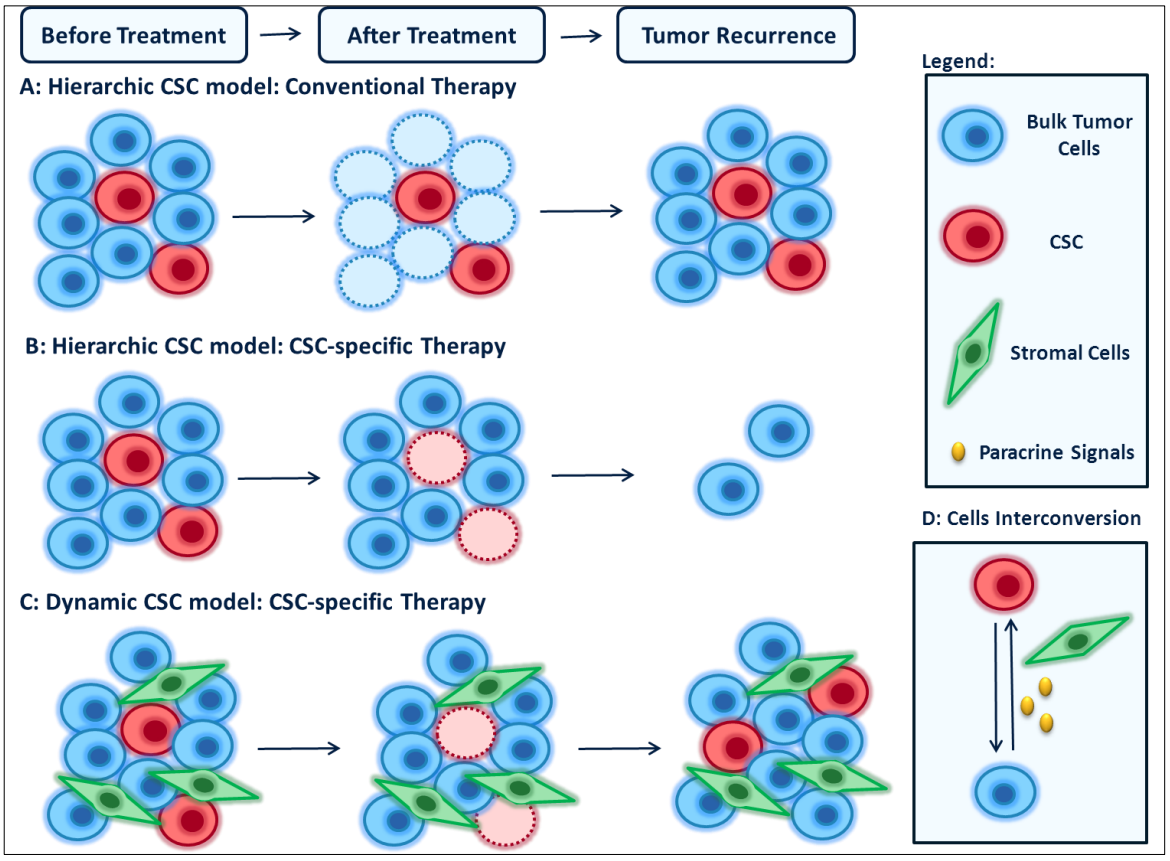


Figure 1. 3. CSC therapy resistance accordingly the different models. (A) CSC are more resistant than tumor bulk cells to most conventional therapeutic interventions. Therefore, after treatment, CSC remain in the tumors or in circulation inducing a rapid recurrence. (B) As for the hierarchic model, CSC specific therapy results in loss of proliferative capacity and decline of the malignancy with tumor regression. (C) Considering the novel dynamic model, the non-CSC can acquire CSC-features through signals from the environment and the stromal cells, thus even with specific CSC eradication a rapid restoration of CSC features occurs, with a subsequent recurrence when therapy is discontinued. (D) The best strategy to tumor remission should target not only the CSC but also the bulk tumor cells as well as the interconversion capacity between them. Stromal cells represent myofibroblasts, endothelial cells, mesenchymal stem cells, or infiltrating immune cells. CSC: cancer stem cells.

In summary, CSC have been considered the major critical player in tumor malignancy and recurrence, thus strong efforts have been recently done to specifically target CSC subpopulation in order to improve therapy efficacy and avoid recurring tumors. However,

recent data suggest that targeting exclusively the CSC sub-populations may not be sufficient because acquisition of stemeness phenotype by other cells within tumors seems to be a bidirectional dynamic process **(Figure 1.3B)** (26, 27). Because the hierarchical model of cancer cannot explain the dynamic behavior of CSC, a new interconversion cancer model has been very recently postulated **(Figure 1.3C)** (26-28). According to the model, the amount of CSC within a tumor or cancer cell line seems to be inconstant and finely tuned, to maintain a specific equilibrium between CSC and non-CSC populations. In one hand, CSC can differentiate to non-CSC which in turn can de-differentiate and revert into cells with CSC properties, like resistance and self-renewal capacity with typical stemness gene expression signature. This cell de-differentiation process was first predicted by Marcov mathematical model (28) and recently confirmed experimentally in various CSC models (28-30). In melanoma, a subpopulation of slow-cycling cells that express histone demethylase JARD1B was identified. As expected from CSC, during propagation of purified JARD1B positive cells appeared a JARD1B negative, non-CSC population. Conversely, a single JARD1B negative cell originates a heterogeneous progeny including JARD1B positive cells, suggesting a bidirectional dynamic of the CSC phenotype (31). Similarly, normal somatic basal-like mammary epithelial cells are able to spontaneously de-differentiate into stem-like cells (32). Further, regeneration of stem-like cells is observed as well *in vivo* after sorting, suggesting that stable equilibrium of CSC/non-CSC cells within a tumor occurs due to cell-state interconversion (28). These findings reinforce the hypothesis of the existence of a controlled balance between CSC and non-CSC populations. It seems that cancer cells might survive to stress conditions by entering de-differentiation as survival mechanism. Several external factors and paracrine communications as well as cell-to-cell interactions are involved in the regulation of this process. As an example, it has been shown that apoptotic cells excrete prostaglandin E2 (PGE2) which promotes proliferation of neighbouring CSC after chemotherapy, whereas PGE2-neutralizing antibodies abrogate CSC repopulation after treatment (33). Similarly, the matrix cellular protein SPARC (secreted protein acidic and rich in cysteine, also known as osteonectin or BM-40) secreted by non-CSC is able to modulates the metastatic capacity of CSC in prostate cancer models (34), and Interleukin-6 (IL-6) secreted by non-CSC is reported to induce formation of breast CSC in association with octamer-binding transcription factor 4 (OCT4) expression (35). Furthermore, OCT4 overexpression is induced via IL-6-JAK1-STAT3 signaling pathway in low attachment conditions as well as *in vivo* to maintain dynamic equilibrium (36).

Therefore, the dynamic phenotype of CSC represents an important challenge for targeted cancer therapies, as tumor cell populations are continuously evolving and therapeutic eradication of existing CSC populations might be followed by their regeneration from non-

CSC. Regarding this, the major goal is to find a way that allows to targeting the CSC but also stopping the interconversion between CSC and non-CSC **(Figure 1.3D)**.

1.2.2. Cancer Stem Cell Properties

Even though the intratumoral amount of CSC as well as the expression of stemness markers among this sub-population differs, depending mostly on tumor types, the essential stemness properties of CSC like self-renewal, tumor initiation capacity and long-term repopulation potential are common features independently of tumor type. Owing their ability to survive in non-attachment conditions showing capacity to grow as tridimensional tumorspheres, CSC have increased capacity to initiate tumor growth *in vivo*, migrate and intravasate the blood stream generating distant metastasis at specific sites. CSC are substantially insensitive to most conventional anticancer therapies, antimitotic agents, and/or radiation (37). Such aggressive behavior results from a particular CSC phenotype as well as their characteristic gene expression and epigenetic modifications. In particular, CSC have a potent machinery of anti-apoptotic proteins and a efficient activation of the DNA damage sensor and repair machinery for maintenance of genome integrity (9, 37). Moreover, CSC overexpress a great number of detoxifying enzymes (aldehyde dehydrogenase 1 (ALDH1A1), bleomycin hydrolase (BLMH)), drug efflux transporters (P-glycoprotein (P-gp)), breast cancer resistance protein (BCRP), and the multidrug resistance-associated proteins (MRP) at the cell membrane, which pump-out chemotherapeutic drugs from the intracellular space (10, 38). In addition, induction of quiescence in stress conditions and higher stability under hypoxic conditions provide CSC with further protection against anticancer therapies (10). Accordingly, the percentage of CSC within a tumor often increases after treatment (33, 37, 39, 40). This often leads to cancer recurrence and metastatic growth since only few CSC are necessary and sufficient for tumor regeneration *in vivo* (41). For all these reasons, if cancer has to be eradicated, new therapies should specifically target the CSC fraction.

1.2.3. Targeting CSC

In agreement with the interconversion cancer model, traditional cancer treatments appear to be effective at reducing tumor mass but often fail to produce desired long-term outcomes, and remission of metastatic disease is common. Moreover, current treatments often render

the tumor resistant at the time of remission, possibly due to their inability to eliminate CSC (8, 37).

Therapeutic targeting of CSC may have the potential to remove residual disease and become an important component of any given cancer treatment. Currently, there are multiple anti-CSC agents in pre-clinical and clinical trials **(Table 1.1)** (42). The likelihood of their clinical success will depend on many aspects, including safety, specificity, pharmacokinetics etc., but also on their ability to effectively target CSC. In fact, most drugs specifically designed to target CSC are based on the effective inhibition of crucial CSC signaling pathways as example: i) cell survival and proliferation pathways; including PI3K-AKT, JAK/STAT, and NF-kB signaling, and ii) signaling linked to the stemness properties of CSC like self-renewal and pluripotency (Notch pathway, WNT pathway and Hedgehog signaling). Of note, one of the risks associated to drugs targeting commonly shared cellular pathways is the difficulty to distinguish normal cells and/or stem cells from CSC. High throughput screening methodologies are reliable approaches to reveal new therapeutic targets that effectively eliminate CSC without compromise the normal somatic stem cells. One of the hopeful targets identified by high throughput screening is Arachidone 5-lipoxigenase (Alox5). Alox5 was found over-expressed in chronic myeloid leukemia CSC but not in normal hematopoietic stem cells (43). It was shown that the absence of Alox5 due to genetic modification or its inhibition with a specific inhibitor, Zileuton, impaired the induction of leukemia in the BCR-ABL inducible cancer model of chronic myeloid leukemia (CML) (43). The inhibition of Alox5 leads to an upregulation of the tumor suppressor gene Msr1 and consequently, to the inhibition of PI3K-AKT and β-catenin pathways (44). Currently, the use of Zileuton in combination with Imatinib is in phase I clinical trials for first line therapy in acute myeloid leukemia. Of note, Imatinib alone does not cure CML because it is not able to kill leukemia stem cells (45) . Another example of a successful high through screening is the chemical screen method for agents with CSC-specific toxicity. As a result, salinomycin was identified as a drug showing selective toxicity for CSC (46).

Previous results from preclinical trials in human xenograft mice models and clinical pilot studies reveal that salinomycin is able to effectively eliminate CSC and to induce partial clinical regression of heavily pre-treated and therapy-resistant cancers, particularly in combination with novel tumor-targeted drugs (47). However, a very important drawback for the potential clinical application of Salinomycin is its marked neural and muscular toxicity (48). In this context, incorporation of cytotoxic drugs within different particulate nanocarrier systems, i.e. liposomes and polymeric particles, is a useful approach to modify the biodistribution of drugs and target tumors. Nanomedicines can in fact address not only the biodistribution profile of CSC-targeting drugs but also reduce their overall toxicity and

enhance their therapeutic efficacy by promoting higher local drug exposure and improving their transport properties across biological membranes (49).

Table 1. 1. Drugs under clinical development with therapeutic activity against CSC*

Therapeutic Target	Drug	Cancer Type	Clinical phase	Trial reference
PI3K, mTORC1/2	VS-5584	Solid tumors	I	NCT01991938
AKT	MK2206	Breast cancer	II	NCT01277757
	GSK2141795	Breast cancer	II	NCT01964924
	AZD5363	Breast cancer	I/II	NCT01625286
STAT3	OPB-31121	Solid tumors	I	NCT00955812
NF-κB	Parthenolid analog	Leukemia	II/III	ISRTN40571019
Multiple kinases	BBI503	Solid tumors	I/II	NCT01781455
STAT3, β-catenine, Nanog	BBI608	Gastric cancer	III	NCT02178956
		Esophageal cancer	III	NCT02178956
		Colon cancer	III	NCT01830621
	BBI503+ BBI608	Solid tumors	I	NCT02432326
WNT pathway	Vantictumab	Solid tumors	I	NCT0 1345201
	Ipafricept	Pancreatic cancer	I	NCT02050178
Notch 2,3 (receptor)	Tarextumab	Pancreatic cancer	II	NCT01647828
		Lung cancer	II	NCT01859741
DLL (Notch ligand)	Demcizumab	Ovarian cancer	II	NCT01952249
		Pancreatic cancer	II	NCT02289898
		Lung cancer	II	NCT02259582
Hedgehog	GDC-449	Solid tumors	II	NCT00739661
ABC transporters	MS-209	Ovarian cancer	II	NCT00739661
	Tariquidar	Ovarian cancer	II	NCT00069160
		Cervical cancer	II	NCT00069160
		Lung cancer	II	NCT00069160
		Kidney cancer	II	NCT00069160
Chemokine receptors 1,2	Reparixin	Breast cancer	II	NCT01861054
IL-3 receptor	SL-401	Leukemia	I/II	NCT02268253
Alox5	Zileuton	Leukemia	I	NCT02047149

*Details including dosage of the chemical agents under trial, duration of the trial, inclusion and exclusion criteria for recruiting patients, contact and locations where the trial is being conducted, and the current status of the trial can be obtained by searching the 'Trial reference' in the US National Institutes of Health Registry (www.clinicaltrials.gov) and EU Clinical Trials Register (www.clinicaltrialsregister.eu). ABC: ATP-binding cassette; AKT: protein (serine/threonine) kinase B (PKB); Alox5: arachidone 5-lipoxigenase; DLL: delta-like ligand; IL-3: interleukin-3; mTORC1/2: mammalian target of rapamycin complex 1 and 2; NF-κB: nuclear factor kappa B; PI3K: phosphoinositide 3-kinase; STAT3: signal transducer and activator of transcription 3; WNT: wingless-related integration site.

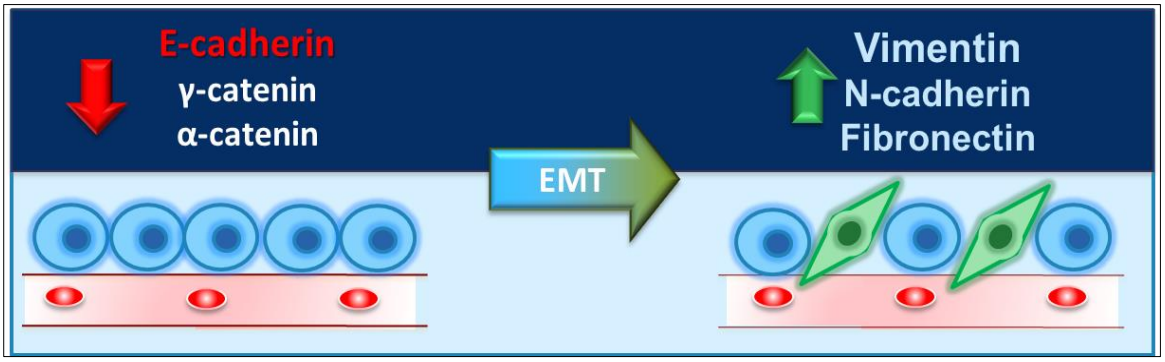


Figure 1. 4. Schematic representation of the EMT process as well as some of the most representative affected proteins. EMT: epithelial-mesenchymal transition.

1.3. Epithelial-Mesenchymal Transition (EMT)

As referred previously, tumor invasion and subsequent metastization are the major causes of morbidity and mortality in patients with cancer (50). Most of malignant tumors are carcinomas originated from various epithelial tissues. EMT is the biological process that triggers the evolution from a well-differentiated adenocarcinoma to an invasive epithelial carcinoma **(Figure 1.4)** (51). It is defined as a phenomenon during which cells lose their characteristic epithelial traits: columnar or polygonal shape, apico-basolateral polarization, organization in cell layers with strong cell-cell adhesion and limited migratory potential. During this event, cells acquire mesenchymal features such as, spindle-shaped morphology, anterior-posterior polarization, focal cell-cell contacts and strong migratory potential, becoming able to detach from their original site, penetrate the bloodstream, and subsequently create metastasis at different sites. These morphological and functional changes, demands several intracellular molecular alterations such as the loss of epithelial markers including E-cadherin, certain cytokeratins, occludin, and claudin, while mesenchymal markers, namely N-cadherin, vimentin and fibronectin are upregulated (52, 53).

EMT is an essential process for appropriate embryonic development. In adults, EMT occurs during wound healing, tissue regeneration, organ fibrosis, and cancer progression (8). Based on the biological context in which EMT occur, this mechanism can be classified into three different subtypes presented in **Table 1.2** (53). During cancer progression, the genetic and epigenetic changes that mainly affect oncogenes and tumor suppressor genes of epithelial carcinoma cells seem to be responsible for the activation of an EMT program (54, 55). Thus, preventing epithelial carcinoma cells to undergo such transition in order to keep the tumor in its primary stage and, therefore, treatable should be a future goal of therapeutic intervention.

Table 1. 2. **Different types of EMT and their main functions.**

Type of EMT	Involved in	Function
EMT 1	Embryogenesis Organ development	Generation of cells with mesenchymal phenotype with potential for the development of new tissues
EMT 2	Tissue regeneration Organ fibrosis	Generation of fibroblasts for tissue reconstruction following a trauma or inflammatory injury
EMT 3	Tumor metastasis	Acquisition of mesenchymal characteristics by epithelial carcinoma cells and loss of cell adhesion molecules. Acquisition of capacity to detach from original site and enter the bloodstream.

EMT: epithelial-mesenchymal transition.

Additionally, the necessity of EMT inhibition in epithelial carcinoma cells is also related with the multidrug resistance (MDR), frequently observed in cells that have undergone EMT. In fact, it was demonstrated that therapeutic strategies intended to modulate EMT pathways have led both to a decrease in invasive potential and to an increase in drug sensitivity (56, 57).

As a transient mechanism, the EMT is reverted when the circulating metastatic cells extravasate through blood or lymphatic compartment to fix and form the distant tumors, being this inverse process called Mesenchymal-Epithelial Transition (MET) (53, 57). Despite remaining a not completely characterized process, the most important step of MET consists in E-cadherin re-expression and cancer cells re-epithelization, being the metastatic lesions and the primary tumors morphologically similar (58, 59). Taking this in consideration, Wang G, *et al*. (2011) raise the hypothesis that E-cadherin may act as a tumor suppressor gene at the primary tumor site but may be considered oncogenic at distant metastatic sites (60).

1.3.1. Adhesion-related Proteins: E-cadherin

Metastatic events require molecular changes that enable the cells to leave the original tumor site. This process involves the disruption of the cell-cell adherent junction complex through E-cadherin downregulation. Also, the upregulation of matrix metalloproteinases and basement membrane degradation enzymes are responsible for improving invasion into the surrounding tissues (61).

E-cadherin is a central component of cell-cell adherent junctions (**Figure 1.5**). Adhesion occurs when E-cadherin bind to other E-cadherin molecules on adjacent cells, forming a homotype adhesion. This mechanism is required for the formation of epithelia in the embryo

and for the maintenance of the epithelial homeostasis in adults. It is a 120 kDa glycoprotein with a large extracellular domain comprising five cadherin-motif subdomains, a single transmembrane domain and a short conserved cytoplasmic domain interacting with several proteins collectively termed catenins. As calcium is essential to mediate adhesive function of E-cadherin, its extracellular portion contains several calcium-binding sites. For intracellular adhesion, cytoplasmic portion of E-cadherin bind tightly to β-catenin, a cytoplasmic protein that is non-covalently linked to α-catenin, which in turn binds to the actin cytoskeleton, either directly or indirectly via actin-binding proteins as alpha-actinin and vinculin (62, 63).

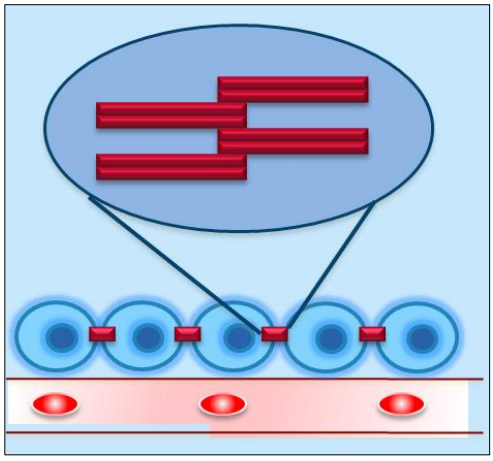


Figure 1. 5. Schematic representation of E-cadherin. E-cadherin being part of the cell-cell adherent junctions, is a crucial player in the cell adhesion and in the maintenance of the epithelial phenotype.

The maximum homotypic activity of E-cadherin results from it linkage to cytoskeleton. Therefore, the reduction of E-cadherin expression promotes a sequence of signaling events and major cytoskeletal reorganization. This reduction of E-cadherin expression in human tumors may be caused by several mechanisms such as i) proteolytic cleavage of the extracellular domain; ii) somatic mutations; iii) homozygous chromosomal deletions; iv) extracellular glycosylation that suppresses hemophilic adhesion; and v) silencing of the CDH1 promoter by DNA hypermethylation or through transcriptional repression (50, 64, 65). E-cadherin gene, CDH-1, located on chromosome 16q221, is a member of a large family of genes coding for calcium-dependent cell adhesion molecules (CAMs). It is a conserved gene, which, apart from its role in normal cells, can play a major role in malignant cell transformation and tumor progression (62, 64).

The downregulation of E-cadherin expression, and the consequent dysfunction in cell-cell adhesion, is a major hallmark of the EMT process and is the main molecular alteration correlated with the invasive and metastatic process in multiple types of cancer, being then

considered a critical tumor suppressor protein (52, 66). Thus, many studies have focused on understanding the interaction between loss of E-cadherin expression and the evolution to an invasive epithelial carcinoma (62, 67).

In fact, many experiments in cancer cell cultures or *in vivo* tumor models have shown that the promotion of E-cadherin expression can revert the ability of several types of carcinomas to invade and metastasize and that, on the contrary, the abrogation of E-cadherin-mediated cell adhesion in noninvasive tumor cells activates their invasiveness and metastatic powers. These studies underline the importance of E-cadherin in the inhibition of tumor invasion and metastasis (58, 68).

E-cadherin loss promotes metastasis not only by enabling cellular disaggregation, but also by inducing multiple downstream transcriptional pathways and a wide range of functional changes in human breast epithelial cells. Onder T, *et al*. (2008) used two different methods to inhibit E-cadherin function: a) a short hairpin RNA (shRNA)-mediated knockdown of E-cadherin that lead to a reduction of over 90% in the protein levels, and b) the production of a truncated form of E-cadherin lacking the protein ectodomain but preserving the cytoplasmatic tail. They observed that the presence of truncated proteins do not result in EMT, once the maintenance of the cytoplasmatic tail of E-cadherin avoid the EMT induction. On the other hand, the significant decrease in E-cadherin expression triggers the EMT process and the acquisition of metastatic ability (66).

Downregulation of E-cadherin is accompanied by upregulation of N-cadherin and its placement on cellular membrane. A “cadherin switching” i.e., reduction of E-cadherin leading to an increased N-cadherin expression (EN-switch), is one of the most important changes involved in the EMT pathways. Both isoforms are used as determinant marker proteins to define the epithelial or mesenchymal state of a cell. Additional support for the relation between tumor malignancy and the ‘‘cadherin switch’’ comes from clinical studies performed by Gravdal K, *et al*. (2007) (69). The study concluded that the occurrence of EN-switch is a more reliable parameter in evaluation of patient prognosis than presence of E-cadherin or N-cadherin separately. The study stressed out the importance of a dependent relationship between the patient prognosis and EN-switch. They also conclude that a ‘‘cadherin switch’’ had a prognostic effect on time to both biochemical failure and clinical recurrence in multivariate survival analyses, stronger than for each protein separately (69).

1.3.2. Tumor-associated Proteins in EMT Activation

1.3.2.1. The influence of tumor microenvironment

The existence of an extracellular matrix in the highly unorganized tumor epithelium brings together cells and secreted proteins to this environment. The SPARC is one of those proteins that belong to the matrix cellular family and appears to be closely related with the E-cadherin expression. It is a nonstructural component of extracellular matrix (ECM) that regulates interactions with several ECM components, modulating the activity of several cytokines and growth factors, promoting alterations in cells adhesion and proliferation capacities (70). Structurally, the SPARC-related proteins subfamily presents three main components: i) an acidic N-terminal domain with low calcium-binding affinity, ii) a disulfide-bonded, copper-binding follistatin domain, with homology to the TGF-β inhibitors and iii) extracellular C-terminal domain with calcium-binding affinity (70). Robert G, *et al*. (2006) reported for the first time the interaction between SPARC and E-cadherin, showing that the overexpression of SPARC in normal human melanocytes give them a fibroblast-like morphology related with the E-cadherin downregulation via the induction of Snail, an E-cadherin transcriptional repressor (71). Conversely, SPARC depletion reduced the levels of Snail promoting the E-cadherin upregulation (71).

The relationship between E-cadherin and SPARC can be understood based on the recently described mechanism of integrin-linked kinase (ILK) stimulation. Due to their localization, integrin receptors seem to be the link between SPARC (extracellulary located at points of integrin-ECM interaction) and ILK (located at the plasma membrane). Subsequently, the phosphorylation of several effector proteins by the ILK, including AKT and integrins-β1/β3, modifies cell behavior in terms of adhesion, invasion and metastization ability (72-74). Nevertheless, SPARC tumorigenicity seems to be dependent on the cell type and the tumor environment. In fact, its overexpression is associated with metastization and invasiveness in melanoma, glioma, breast, prostate and colorectal carcinomas, while in the case of ovarian carcinoma, neuroblastoma and pancreatic adenocarcinoma its expression is inversely related with the tumor development (70, 71, 75).

Still at the extracellular level, the early observations also suggested that tumor cells bearing a mesenchymal phenotype massively secrete active TGF-β, which triggers EMT and enhances angiogenesis in the tumor microenvironment. In early tumor development, TGF-β has an inhibitory effect on tumor growth, acting as a tumor suppressor. Conversely, at later stages, it acts as an immunosuppressive cytokine, favoring tumor evasion, by converting $CD4^+$T cells in immunosuppressive T regulatory cells and inhibiting the effector T cells functions (76). Tumor cells autocrine TGF-β production aims to circumvent tumor

microenvironment immunity system, mainly by suppressing dendritic cells development, maturation and function (65, 77, 78).

The cellular responses to TGF-β begin through the activation of the membrane receptor kinases complex and occur via activation of members of Smad family. Smad 2, 3 and 4 form a complex that translocate to the nucleus and bind to transcriptions factors, triggering the repression of epithelial genes and the concomitant activation of mesenchymal genes. Snail expression, for instance, is activated when Smad3 binds to Snail promoter. Consequently, E-cadherin expression is suppressed and vimentin and fibronectin expression enhanced. Silencing Snail expression reverses this process and restores E-cadherin levels (77, 79).

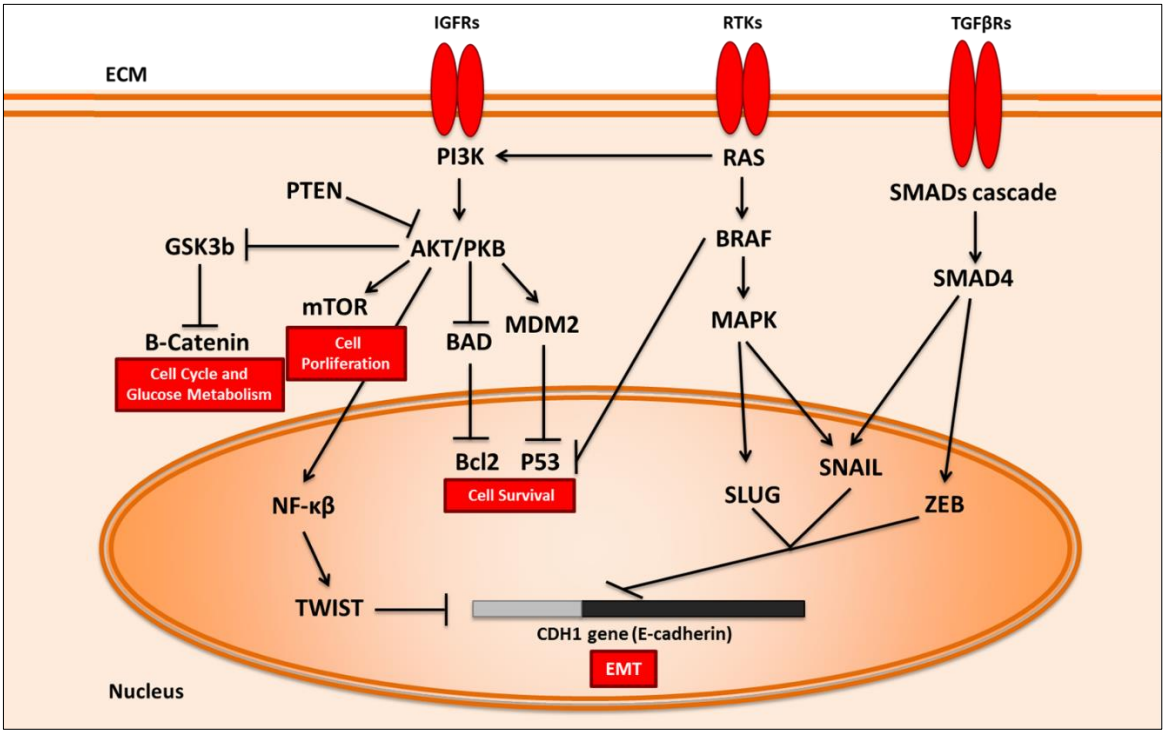


Figure 1. 6. Schematic representation of the most important biological pathways involved in cancer and EMT activation. AKT: protein (serine/threonine) kinase B (PKB); BAD: Bcl2-associated death promoter; Bcl2: B-cell lymphoma 2; ECM: extracellular matrix; EMT: epithelial-mesenchymal transition; GSKβ3: glycogen synthase kinase 3 beta; IGFR: insulin-like growth factor receptor; MAPK: mitogen-activated protein kinase; MDM2: mouse double minute 2 homolog; mTOR: mechanistic target of rapamycin; NF-κβ: nuclear factor kappa B; PI3K: phosphoinositide 3-kinase; RTK: receptor tirosine kinase; SLUG: zinc finger protein SNAIL2; SNAIL: zinc finger protein SNAIL1; TGFβR: tumor growth factor beta receptor; ZEB: zinc finger E-box-binding homeobox.

TGF-β induced EMT is dependent on several other pathways, such as the MAPK/ERK pathway and also requires the activation of transcription factors such as nuclear factor kappa B (Nf-κB) (65). RAS, upstream of the MAPK/ERK pathway is active in many tumors via stimulation by receptor tyrosine kinases (RTKs) or by oncogenic mutations. Ras-MAPK

pathway induces the expression of important EMT-related transcription factors, such as Snail and Slug. Besides MAPK pathway, Ras also activates other important downstream pathways such as the Phosphatidyl-inositol-3'-kinase (PI3K) signaling pathway, explained later on in this chapter (65, 78, 80). The Nf-κB factor has been associated with tumorigenesis due to its anti-apoptotic effect in several cancer cells. Nf-κB activation, commonly observed in metastatic breast cancer cells, occurs in response to inflammatory cytokines and growth factors. It has shown to be a fundamental regulator of EMT and metastasis in a model of breast cancer progression, once Nf-κB hyperactivation seems to be related with the increased expression of TWIST in these cells (65, 78, 81).
The referred pathways involved in EMT regulation are summarized and schematized in **Figure 1.6**.

1.3.2.2. Players at the cell membrane level

Apart from extracellular signals, the EMT mechanisms also require important players in the cell membrane. Early observations suggested that the integrin's family is implicated in cell adhesion mechanisms. These heterodimeric transmembrane proteins containing one α- and one β- subunit are involved in the crosstalk between the tumor cell and ECM, with many implications in crucial cellular processes such as cell proliferation, differentiation, migration, invasion and apoptosis. During the EMT, changes in the ECM microenvironment or alterations in intracellular signals that affect the expression of integrin-associated proteins might cause alterations in integrin activity. Consequently, changes in the overall adhesive and migrative status of the cells are expected to occur. Studies on integrin-β1 and its correlation with histological grading of cancer metastasis have insinuated integrin-β1 as a potential therapeutic target in metastatic cancer (50). However, the study of Parvani JG, *et al*. (2013) demonstrated that the specific downregulation of integrin-β1 failed to affect the growth and dissemination of breast cancer due to the compensatory upregulation of integrin-β3, which drives EMT through the oncogenic TGF-β signaling pathway. Therefore, these findings highlight the importance of targeting both β1 and β3 integrins to more efficiently eradicate breast cancer metastatic disease (82).
The well-known relation between integrins and TGF-β in the stimulation of EMT and metastasis renders integrin-β1 to be an important player in several key signaling cytoplasmic transduction pathways (83).
Additionally, several reports linked the integrin-β1 induced signals with the AKT isoforms activation explained below. As an example, Arboleda MJ, *et al*. (2003), demonstrated that AKT2 causes the upregulation of integrin-β1, increasing human breast and ovarian cancer

cells invasion and metastization (84). More recently, Virtakoivu R, *et al*. (2012) demonstrated *in vitro* that in prostate cancer cells, both AKT1 and AKT2 are inhibitors of integrin-β1. In this model their downregulation promotes the activation of cell surface integrin-β1, enhancing migration and invasion (85). Taken together, these results indicate that AKT may have a major role in the regulation of cell motility and invasiveness, being therefore a possible prognostic factor to identify the patient metastization stage. However, other EMT players present similar behavior, stressing out that the effect of integrins activation is both cell-type and differentiation-stage dependent (85-87).

1.3.2.3. Intracellular transcriptional regulation of EMT

E-cadherin downregulation, crucial to the initiation of an EMT process, requires genes downregulation followed by the activation of mesenchymal oncogenes expression. Identification of these genes could lead to development of new tumor markers and new strategies for targeted therapy.

The transcriptional regulation of EMT essentially involves three families of transcription factors, the Snail, ZEB and bHLH families (50, 58, 65). The zinc-finger transcription factors Snail, Slug, ZEB1 (δEF1), ZEB2 (SIP1) and E47, a member of the bHLH family, directly bind to the E-boxes of the E-cadherin promoter, repressing its transcription (88). TWIST-based signaling pathways also heavily influence loss of E-cadherin expression. However, as explained below, it is not clear yet how TWIST, another member of the bHLH family, promotes the E-cadherin downregulation. Nevertheless, the effect of the different transcription factors in E-cadherin expression highly depends on the histological type of tumors, not being present or active in all cancer cells (55).

1.4. The Importance of TWIST-based Signaling Pathways in the EMT Program

Due to its recognized importance in EMT activation, a major attention is given to the transcriptional regulation of E-cadherin by the transcription factor TWIST, and the intracellular pathway that this putative oncogene regulates (89). TWIST and a wide range of TWIST-related proteins and genes have been extensively reported as targets for a future anticancer gene therapy.

TWIST is a key transcription factor that regulates E-cadherin expression, induces EMT and promotes cell motility. It has a specific role activating the migratory machinery and facilitating the invasion step of metastasis (50, 56, 58). Yang J, *et al*. (2004) identified the

transcriptional factor TWIST as being an essential player in metastization (90). In order to understand how the inhibition of TWIST expression affects the metastatic ability of the highly metastatic cell lines (4T1 cells), siRNAs targeting TWIST were constructed to further investigate its effects in lung metastases *in vivo*. The results of this experiment showed that the number of lung metastasis was significantly reduced in animals treated with TWIST-siRNA, comparing with a high metastization level observed in the group transfected with the siRNA control sequence. Authors also induced TWIST expression in MDCK kidney epithelial cells and observed morphological and molecular alterations that characterize EMT. Namely, cells lost their cobblestone appearance and acquired a fibroblastic morphology whereas at the molecular level, expression of adherent junction proteins such as E-cadherin, α- β- and γ-catenins was inhibited and mesenchymal markers strongly increased (90).

Despite the above described TWIST interaction with the E-cadherin promoter remains unclear. Thiery JP, *et al*. (2009) and Yang J & Weinberg RA (2008) support that TWIST repress E-cadherin transcription indirectly (56, 58). Conversely, Vesuna F, *et al*. (2008) demonstrated that TWIST binds to the E-cadherin promoter and subsequently transcriptionally represses E-cadherin expression in breast cancer cells (89).

Importantly, TWIST overexpression is also related with chemotherapy failure due to acquired drug resistance. Wang X, *et al*. (2004) described that upregulation of TWIST was associated with cellular resistance to Taxol® in nasopharyngeal, bladder, ovarian and prostate cancer cell lines. This suggests that inactivation of TWIST expression in human cancer cells, could represent an alternative therapeutic strategy to overcome the acquisition of paclitaxel (PTX) resistance (91). In addition to its interference with microtubules and the consequent mitotic arrest, the cytotoxic effect of PTX occurs through multiple pathways related to apoptosis induction. TWIST anti-apoptotic function can occur by i) interfering with p-53 related pathways; ii) inhibition of $p21^{waf1}$ via both p53-dependent and independent pathways and iii) modulating NF-κB pathways (91).

Drug resistance was proved to be related with EMT process and E-cadherin impairment, since migratory and metastatic potential appeared increased in PTX-resistant cells, which also showed decreased expression of E-cadherin and increased expression of vimentin, fibronectin and EMT-regulatory transcription factors, such as Snail and TWIST. In addition, highly tumorigenic cells able to evade apoptosis through an increase of AKT2 expression with a consequent TWIST transcriptional activity stimulation have proved to become drug resistant. Nevertheless, this association between EMT and PTX-resistance should continue to be studied in order to develop therapeutic alternatives in cancer therapy.

1.4.1. TWIST Mechanism of Action: AKT2 and PI3K as the Leading Players

Specially related to the EMT program is the TWIST capability to target an important group of proteins, the AKT (92, 93). AKT 1, 2 and 3 isoforms (or PKB α, β and γ) are some of the most frequently overexpressed kinases in epithelial human cancers, playing a crucial role on tumor initiation, progression and metastasis formation (94, 95). The three isoforms are structurally homologous and present identical mechanisms of activation generally triggered by the stimulation of cell growth factors receptors such as the insulin-like growth factor-I receptor (IGF-IR) (95-97). PI3-K is one of the signaling proteins that is activated downstream of IGF-IR and lies upstream of AKT. PI3-K catalyzes the phosphorylation of membrane inositol lipids (PIs) generating second messengers (3-PIs) that activate the downstream target proteins by binding to their pleckstrin homology domain (PH) **(Figure 1.7)**.

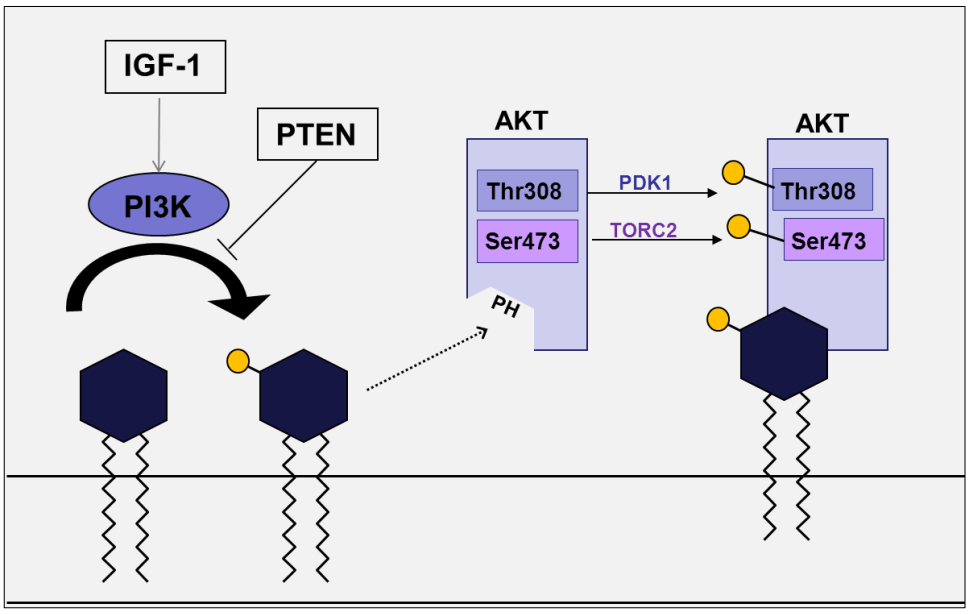


Figure 1. 7. Schematic representation of PI3K/AKT2 mechanism of action. When activated by IGF-1, PI3K phosphorylates its substrate, phosphatidylinositol at the 3 position hydroxyl group of the inositol ring. Phosphorylated PI3 binds to pleckstrin domain of AKT, which can be activated by phosphorylation of Ser and Thr residues. Once activated, AKT can exert its kinase activity on various targets in the cell.AKT: protein (serine/threonine) kinase B (PKB); IGF-1: insulin-like growth factor 1; PDK1: 3-phosphoinositide-dependent protein kinase 1; PH: pleckstrin homology domain; PI3K: phosphoinositide 3-kinase; PTEN: phosphatase and tensin homolog; Ser: serine; Thr: threonine; TORC2: target of rapamycin complex 2.

All AKT isoforms, the major downstream effector of PI3-K, present this PH domain constituted by about 100 amino acids in their N-terminal region. Therefore, due to its PH domain, AKT is recruited to specific sites on plasma membrane containing increased PIs and subsequently is activated by phosphorylation. After its activation, AKT is able to phosphorylate several proteins inside the cell, regulating multiple biological functions related to cancer progression (95, 96).

The importance of PI3-K/AKT signaling pathway has been recognized as a key player in processes involved in tumor development as well as chemoresistance. A detailed knowledge of the structure, regulation and function of PKB/AKT family is nicely reviewed by Hanada M, *et al*. (2004) (98). Singularities between AKT isoforms can be found both on their biological activity and tissue distribution. AKT1 and AKT2 are ubiquitously expressed, while AKT3 present a more limited distribution, with preferential expression in brain and testis (99).

Some reports have shown that the overexpression of a specific AKT isoform could be related with certain type of tumors. For instance AKT1 gene amplification has been associated with gastric and lung cancers. AKT2 has been presenting a critical role in invasion and metastization of human breast, pancreas, colon and ovarian cancer cells, while AKT3 expression has been related with melanoma, brain and steroid hormone-insensitive carcinomas (84, 100, 101).

The specific role of each AKT isoform in terms of cell motility and invasion is strongly dependent on the type of cancer, and may present opposite effects.

Even more complex is the relative contributions of the different AKT isoforms in the different steps of tumor development; however this lack of functional redundancy is still not well understood (102). As an example of this, Dillon RL, *et al*. (2010) using EMbB2 and PyVmT tumor models demonstrated that AKT1 and AKT2 perform opposite roles in mammary tumorigenesis and metastastization. AKT2 expression did not affect latency of mammary tumor, but has a crucial role triggering invasion and lung metastization in both models. Conversely, activated AKT1 promotes mammary tumor development but impairs metastastization, attenuating cancer cell migration (103). In the case of Linnerth-Petrik, *et al*. (2014) the AKT1 ablation significantly delays initiation of lung tumor growth, whereas AKT2 deficiency dramatically accelerates tumorigenesis in a viral oncogene-induced mouse model of lung cancer and AKT isoform-specific knockout mice (102). On the other hand Attoub S, *et al*. (2015) showed that AKT2 silencing reduced the cellular motility and invasion *in vitro* as well reduction of the tumor growth *in vivo*, showing that AKT2 plays an important role in lung cancer progression and can be an interesting target for lung cancer therapy (104).

This non-overlapping and sometimes opposing function of AKT isoforms leads to some contradictory statements in the literature. Therefore, the consequences of AKT genes overexpression in epithelial human cancers are critical to determine the way AKT inhibitors should be used in cancer treatment (105). A state of art of AKT isoforms silencing studies is present in the next section, aiming to understand their specific role in oncogenesis and the clinical application potential of AKT silencing based therapies.

Regarding the cancer types we were working with, major attention was given to the AKT2 isoform. AKT2 expression undergoes positive transcriptional regulation by the putative oncogene TWIST, being a critical player in the TWIST-promoted metastatic process by enhancing cell migration/invasion. This suggests that TWIST/AKT2 axis regulates the E-cadherin silencing in tumorigenic cancer cells (84, 92, 97). Cheng G, *et al*. (2007) investigated their functional connection in 14 highly invasive breast cancer cells and concluded that AKT2 is a major downstream target of TWIST playing a crucial role in this cells mediated drug resistance, migration and invasion ability. Up to now, their studies demonstrated that i) TWIST binds to a specific E-box and activates the transcriptional activity of AKT2 promoter gene and positively regulates AKT2 expression; ii) TWIST knockdown by siRNA significantly decrease AKT2 expression; iii) ectopic expression of TWIST increase AKT2 at protein and mRNA levels in a dose depend manner; iv) siAKT2 significantly decreased TWIST promoted drug-resistance, invasion and migration; and v) re-expression of AKT2 rescue the effects of TWIST knockdown. The clinical significance of these results *in vivo* was assessed, and it was observed that TWIST-AKT2 co-expression increased 69% in late stage breast tumors (92, 93). It was also demonstrated that, while AKT2 knockdown has a strong effect on EMT morphological changes, the knockdown of TWIST had no significant effect, being the AKT2 the critical player in all the TWIST-mediated effects.

Moreover, AKT2 oncogene is frequently related with cell survival mechanisms mainly in response to stress conditions. According to Arboleda MJ, *et al*. (2003), this protein is crucial in PI3-K dependent signaling pathways involved in cancer cells motility, invasion and metastization (84). Regarding cell survival, generally activated AKT2 promote apoptosis inhibition by different mechanisms such as i) increasing Bcl-2 like anti-apoptotic factors (Bcl-2 or Bcl-xL); ii) direct phosphorylation of Bad and Bax, leading to the inhibition of both pro-apoptotic factors with blockage of cytochrome c release from mitochondria; iii) phosphorylation of NF-κB with consequent transcriptional activation of NF-κB-regulated anti-apoptotic genes; iv) Inhibition of p53 and $p21^{WAF1/CIP1}$; v) inactivation of forehead transcription factors; and vi) promotion of MDM2 nuclear localization and stabilization (92,

96, 106, 107). Thus, phosphorylation of AKT2 may hence prevent programmed cell death process.

For all these, AKT2 became an interesting target to work with, and appear as a relief in the control of tumor development and metastization.

The RNAi mediated AKT2 silencing strategy might prompt TWIST downregulation, avoiding the undesirable TWIST-mediated effects, while restoring E-cadherin expression and reverting the EMT process. Indeed, silencing of the AKT2 isoform has already shown to impair cell chemotaxis, crucial during cellular intra- and extravasation, through the blockage of actin polymerization, impairment of cytoskeletal rearrangement and/or cell adhesion crucial to mobility of metastatic cells (36, 108). Moreover, AKT2 has been shown to be involved in the activation of β-catenin and CD44 expression, fostering the preservation of the CSC subpopulation and mesenchymal traits (92, 109, 110). The involvement of AKT in maintenance of CSC subpopulation within the tumor by regulating EMT via TWIST activation is of special interest.

This strategy could have important consequences in the design of new cancer therapeutic strategies, and special in CSC directed therapeutics.

1.5. RNA Interference Regulation of EMT

Gene therapy is a fascinating strategy that has detained the attention of several researchers in the last years since it allows the modulation of aberrant cell pathways, and to overcome the high toxicity and/or low specificity of many chemical drug compounds. Additionally, many putative protein targets are “non-drugable” due to their inconvenient cellular localization, binding and function (i.e. transcription factors). Consequently, gene therapy has been increasingly demanded nowadays. The silencing of particular genes through the RNAi therapy has been extensively investigated as a promising strategy in the oncological field, given the possibility of targeting specific oncogenes involved in proliferation, survival, angiogenesis, metastasis, apoptosis suppression or drug-resistance (111, 112).

RNAi technology that awarded Fire and Craig Mello the Nobel Prize of Physiology and Medicine in 2006, is a potent technology based on direct homology dependent post-transcriptional gene silencing. The RNAi-based therapeutics can be classified accordingly to their mechanism of action. There are mainly three types that include shRNA (short hairpin RNA), miRNA (micro RNA) and siRNA (small interfering RNA). The differences between them are summarized in **Table 1.3**.

Table 1. 3. Main characteristics and differences between miRNA, siRNA, and shRNA.

	miRNA	siRNA	shRNA
Precursor	70nt dsRNA or shRNA	Long dsRNA or shRNA	Created in cell nucleus
Origin/Delivery to the cell	Occurs naturally in plants and animals. Encoded by their own genes	Via viral and other gene therapy vectors to the cytoplasm	Via viral and other gene therapy vectors to the nucleus
Complementary	Not exact. A single mRNA may target hundreds of mRNA	Perfect match (higher than the shRNA)	Perfect match (lower than the siRNA)
Dose/Administration	-	High/Local or limited systemic injection	Low/Local and systemic injection
Function	Inhibit translation of mRNA	Cleave complementary mRNA	Cleave complementary mRNA
Persistence	-	99% degraded after 48 hours. The silencing is effective up to 5-7 days post-transfection	Expressed for up to 3 years
Main clinical applications	Expression levels of miRNAs can be used as diagnostic and biomarker tools.	Therapeutic agents for acute disease conditions where high doses are tolerable.	Therapeutic agents for chronic, life threatening diseases where low doses are desirable.

shRNA are short hairpin expression vectors that after the nuclear entry, transcribe to small RNA molecules that bind to complementary mRNA and inhibit the expression of corresponding protein. From the delivery perspective, shRNA delivery is the most challenging of the three, since it requires entering the nucleus which also makes it less efficient in slowly dividing and non-dividing cells (113). Since, shRNA is produced inside the target cell from a DNA construct that has been delivered to the nucleus; the silencing effect is persistent during a long period of time (up to 3 years). Contrary to shRNA, miRNA and siRNA are small 21–25 base nucleotides that exert its function directly in the cytoplasm where they interfere with the target mRNA translation process. miRNA inhibits this translation process by binding non-specifically to the 3′UTR (untranslated region) of the mRNA, therefore miRNAs are effective against more than one mRNA with similar sequences. However as a consequence, miRNA are less specific and more indiscriminate, usually presenting undesired off-target activity. On the other hand, siRNA binds specifically to a region of the complementary mRNA knocking down the expression of a certain protein (114-116).

Despite the therapeutic potential associated to the RNAi technology, this strategy is still facing the challenge of poor *in vivo* delivery, uncontrolled off-target effects, and undesired immune system modulation associated to the use of viral vectors (117, 118). In order to

overcome such problems, gene delivery systems based in nanotechnology have been developed and proposed, as further discussed in **Chapter 2**.

Despite the existence of several forms of small RNAs, siRNA and miRNA are the most commonly used for RNAi therapeutic and diagnosis applications (119, 120). Thus, most attention will be given to these two strategies. The mechanism of RNAi associated to both is present in **Figure 1.8**.

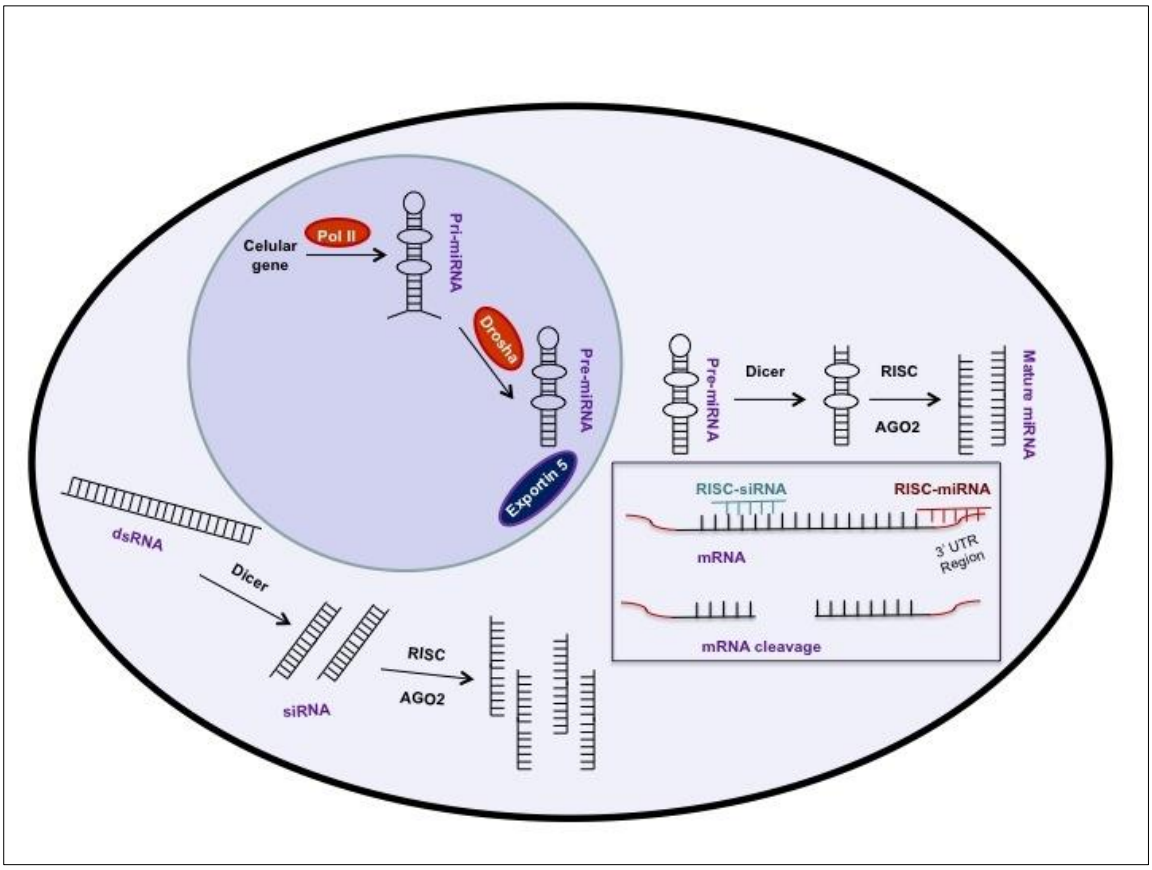


Figure 1. 8. RNA interference mechanism. For siRNA the process begins with the cleavage of a long double-stranded RNA (dsRNA) by the Dicer enzyme complex into small 21-23 nt double stranded RNAs. These siRNAs are incorporated into a nuclease-containing multi-protein complex RNA-induced silencing complex (RISC). RISC is activated when Argonaute 2 (AGO2), the catalytic component of RISC, cleaves the sense strand of the double strand siRNA. Afterwards, RISC containing the guide strand binds to the mRNA inducing an endonucleolytic cleavage within the target site that is also carried out by AGO2. The miRNA mechanism begins with primary miRNA transcripts (pri-miRNAs) that are transcribed by the RNA polymerase II (Pol II). These pri-miRNAs are then cleaved by the Drosha enzyme complex into precursor miRNAs (pre-miRNAs) that are then exported to the cytoplasm by the exportin 5 and incorporated into the Dicer enzyme complex. Dicer will process the pre-miRNA for loading onto the RISC complex. When the RNA duplex is loaded onto RISC the sense strand unwinds, leaving a mature miRNA bound to RISC. This miRNA recognizes target the 3'-UTR sequences in the mRNA and inhibits the mRNA translation.

1.5.1. miRNA and Cancer

The miRNA, such as other conventional cancer genes, appears over- or sub-expressed accordingly to the types of human and animal tumors. Friedman JM, *et al*. (2009) reported that more than half of the total mRNA species encoded in the human genome are controlled by miRNAs (121). Moreover, usually, one miRNA is able to modulate the expression of a wide range of targets. As a consequence of this, when miRNA activity is affected, significant biological consequences are expected. In fact, some human diseases such as cardiac disorders and cancer had been related with modifications in the expression of a given miRNA (122, 123). As an example, miRNA families, such as the miR-200 and miR-29, become a subject for intense scientific investigation regarding their potential as new therapeutic targets and biomarkers for certain diseases (124, 125).

As recently reported, miR-200 regulates E-cadherin expression through its interaction with the mRNA of the transcriptional factors ZEB 1 and ZEB 2. It was proved that the inhibition of miR-200 reduced the E-cadherin expression and increased vimentin, triggering cell EMT events. Additionally, when miR-200 is overexpressed, E-cadherin is upregulated and consequently cell motility is reduced. With regards to these findings, was suggested the use of miR-200 as a biomarker for the E-cadherin positive/vimentin negative cancer cells, and as a potential regulator of the EMT process (126). More interestingly, miRNA-200 family was related with the expression of each AKT isoforms. A decrease in the intracellular concentration of miRNA-200 was observed when the ratio of AKT1 to AKT2 was reduced, resulting in the EMT triggering and, a stem cell-like phenotype acquisition in breast cancer cells (124).

Also extensively explored as potential biomarkers or new targets for cancer treatment, due to their preponderant role in different types of cancer, is the miR-29 family, constituted by the miR-29a, miR-29b and miR-29c. In fact, the members of this family usually show tumor suppressing behavior influencing the expression of several transcriptional factors and cell-matrix adhesion-related proteins such as integrin-β1**.** Consequently, their downregulation triggers abnormal migration, invasion and proliferation of cells (125, 127).

1.5.2. siRNA-mediated Silencing of AKT Isoforms

In the last years, several studies have resorted to the RNAi technology to silence the AKT isoforms in order to understand their specific effects in carcinogenesis. Cheng JQ, *et al*. (1992) were the first who identified AKT gene alterations in human epithelial cancers. They demonstrated that the AKT2 is overexpressed in human ovarian carcinomas (128). Later,

these authors reported the same overexpression in human pancreatic cancer cells. After PANC1 cells transfection with siAKT2 a marked reduction of their tumorigenecity was observed, confirming the role of AKT2 overexpression in the malignant phenotype acquisition (129). The importance of AKT2 as an EMT promoter were also strengthened by Pu P, *et al*. (2006) who investigated the specific effect of AKT2 inhibition in a malignant glioma progression. siAKT2 transfected glioma cells presented both a marked reduction of cell proliferation and apoptosis induction. The *in vivo* studies showed a notable increase in the mean survival rate of the treated animals (130). Irie HY, *et al*. (2005) also silenced AKT1 and AKT2 isoforms in IGF-IR overexpressing breast epithelial cells and assessed its individual effects on the displayed phenotypes. The results obtained indicate that due to AKT1 specific role in inhibiting IGF-I-mediated cell migration and ERK signaling pathway, its downregulation enhances migration and EMT markers expression. In contrast, downregulation of AKT2 inhibits migration and reverts the morphological changes induced by AKT1 downregulation (97).

The study of Virtakoivu R, *et al*. (2012) about the roles of the different AKT isoforms in integrin-β1 regulation represents a good example of the cell type dependent AKT activity. They show that for prostate cancer both AKT1 and AKT2 negatively regulate cell migration and invasion. Therefore, when AKT1 and AKT2 are silenced, cell surface integrin-β-1 is activated, favoring tumor development. They also reported that the mechanism by which each AKT isoform regulates the integrin-β1 activity is different. AKT1 silencing stimulates integrin-β1 via a feedback downregulation of several receptor tyrosine kinases expression. On the other hand, the AKT2 silencing induces the overexpression of miRNA-200 family, which in turn is able to activate integrin-β1 and promote cell migration in the PC3 cells (85). Besides the well-known importance of AKT in the EMT program, the involvement of AKT isoforms in the acquired resistance of cancer cells to anticancer treatment have also been explored. Regarding this, Rajput, S. *et al*. (2015) defend that the silencing of AKT through siRNA provides new therapeutic options against the resistance to traditional chemotherapeutics. In their study they use acidic milieu-sensitive multilamellar gold niosomes (Nio-Au) to targeted delivery of AKT-siRNA and thymoquinone (TQ) to tamoxifen-resistant and AKT-overexpressing MCF7 breast cancer cells. With this strategy of co-delivery of siRNA and a chemical compound, they achieved to effectively knockdown AKT, thereby sensitizing cells to TQ and induce apoptosis of cancer cells. This result was confirmed by *in vivo* experiments (131).

1.6. Conclusions

Despite progress observed in the last decades, an appropriate cure for advanced cancer was not yet achieved. Conventional treatment resistance, metastatic growth and tumor recurrence are sustained by the presence of CSC within the tumor. Due to the self-renewal capacity, CSC represents the essential part of the tumor, while division of any other cell will finish by clonal exhaustion. The percentage of CSC within a tumor often increases after chemotherapy or radiation which leads to cancer recurrence and metastatic growth since only few CSC are necessary and sufficient for tumor regeneration. Therapeutic selective targeting of CSC (specific pathways and selective CSC drugs/siRNA) may have the potential to remove residual disease and become an important component of any given cancer treatment. Currently, there are multiple anti-CSC agents in pre-clinical and clinical trials. Among the different therapeutic options, RNAi technology emerged as a tool to silence the expression of specific genes. The use of siRNAs or miRNAs in gene therapy to reduce the expression of a wide range of oncogenes has been extensively suggested as a new therapeutic strategy in cancer. Nevertheless, this ambitious goal is conditioned by the need of reliable systems for an efficient intracellular delivery.

EMT signaling pathways and its upstream and downstream regulators have gained importance as therapeutic targets for cancer treatment, once they shown to be involved in cell adhesion and disruption, migration, metastization, angiogenesis, apoptosis, and drug resistance. Loss of E-cadherin is recognized as a hallmark of EMT since is a decisive signal for cell invasiveness properties acquisition; however, the network of the involved mechanisms highlights the importance of other crucial players. The enormous complexity of EMT regulators lies in the fact that their effects and expression always depend on the stage of cancer progression and tumor type, sometimes having completely opposite roles during the EMT and MET. This explains the inconsistencies found in different studies on the role of a certain gene in tumor progression. In order to be used as therapeutic target, it is of utmost importance to identify specific EMT and MET regulators for each cell and tumor type since a generalization that comprises all tumors simply does not exist. Special attention has been given to the transcriptional regulation of EMT by TWIST and dependent pathways. Due to its protagonism in malignant progression and in regulating TWIST mediated effects such as intracellular regulation axis in the decrease of E-cadherin expression, AKT2 was identified as a promising therapeutic target to revert mesenchymal phenotype in tumorigenic epithelial carcinoma cells.

1.7. References

1. Organization WH. Cancer http://www.who.int/cancer/en/ [
2. PhRMA. Medicines in development for cancer. www.phrma.org; 2015.
3. American Cancer Society. Cancer facts & figures 2016. www.cancer.org; 2016.
4. Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ. Cancer statistics, 2007. CA: a cancer journal for clinicians. 2007;57(1):43-66.
5. Organization WH. GLOBOCAN 2012: estimated cancer incidence, mortality, and prevalence woldwilde in 2012 http://globocan.iarc.fr/ [
6. EvaluatePharma. World Preview 2015, Outlook to 2020. www.evaluatepharma.com; 2015.
7. Mariotto AB, Yabroff KR, Shao Y, Feuer EJ, Brown ML. Projections of the cost of cancer care in the United States: 2010-2020. J Natl Cancer Inst. 2011;103(2):117-28.
8. Arnedos M, Bihan C, Delaloge S, Andre F. Triple-negative breast cancer: are we making headway at least? Ther Adv Med Oncol. 2012;4(4):195-210.
9. He YC, Zhou FL, Shen Y, Liao DF, Cao D. Apoptotic death of cancer stem cells for cancer therapy. Int J Mol Sci. 2014;15(5):8335-51.
10. Vinogradov S, Wei X. Cancer stem cells and drug resistance: the potential of nanomedicine. Nanomedicine (Lond). 2012;7(4):597-615.
11. Shervington A, Lu C. Expression of multidrug resistance genes in normal and cancer stem cells. Cancer Invest. 2008;26(5):535-42.
12. Gottesman MM, Fojo T, Bates SE. Multidrug resistance in cancer: role of ATP-dependent transporters. Nat Rev Cancer. 2002;2(1):48-58.
13. Husain SR, Han J, Au P, Shannon K, Puri RK. Gene therapy for cancer: regulatory considerations for approval. Cancer Gene Ther. 2015;22(12):554-63.
14. Verma RK, Yu W, Singh SP, Shankar S, Srivastava RK. Anthothecol-encapsulated PLGA nanoparticles inhibit pancreatic cancer stem cell growth by modulating sonic hedgehog pathway. Nanomedicine. 2015;11(8):2061-70.
15. Tufts Center for the Study of Drug Development. Personalized Medicine Gains Traction but Still Faces Multiple Challenges. www.csdd.tufts.edu; 2015.
16. PhRMA. Medicines in development - biologics. www.phrma.org; 2013.
17. Nowell PC. The clonal evolution of tumor cell populations. Science. 1976;194(4260):23-8.
18. Fearon ER, Vogelstein B. A genetic model for colorectal tumorigenesis. Cell. 1990;61(5):759-67.

19. Lengauer C, Kinzler KW, Vogelstein B. Genetic instabilities in human cancers. Nature. 1998;396(6712):643-9.

20. Wicha MS, Liu S, Dontu G. Cancer stem cells: an old idea--a paradigm shift. Cancer Res. 2006;66(4):1883-90; discussion 95-6.

21. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med. 1997;3(7):730-7.

22. Reya T, Morrison SJ, Clarke MF, Weissman IL. Stem cells, cancer, and cancer stem cells. Nature. 2001;414(6859):105-11.

23. Darini CY, Pisani DF, Hofman P, Pedeutour F, Sudaka I, Chomienne C, et al. Self-renewal gene tracking to identify tumour-initiating cells associated with metastatic potential. Oncogene. 2012;31(19):2438-49.

24. Clevers H. The cancer stem cell: premises, promises and challenges. Nat Med. 2011;17(3):313-9.

25. Shackleton M, Quintana E, Fearon ER, Morrison SJ. Heterogeneity in cancer: cancer stem cells versus clonal evolution. Cell. 2009;138(5):822-9.

26. Shackleton M. Normal stem cells and cancer stem cells: similar and different. Seminars in cancer biology. 2010;20(2):85-92.

27. Kreso A, Dick JE. Evolution of the cancer stem cell model. Cell Stem Cell. 2014;14(3):275-91.

28. Gupta PB, Fillmore CM, Jiang G, Shapira SD, Tao K, Kuperwasser C, et al. Stochastic state transitions give rise to phenotypic equilibrium in populations of cancer cells. Cell. 2011;146(4):633-44.

29. Lu H, Clauser KR, Tam WL, Fröse J, Ye X, Eaton EN, et al. A breast cancer stem cell niche supported by juxtacrine signalling from monocytes and macrophages. Nat Cell Biol. 2014;16(11):1105-17.

30. Sharma RB, Wang Q, Khillan JS. Amplification of tumor inducing putative cancer stem cells (CSCs) by vitamin A/retinol from mammary tumors. Biochem Biophys Res Commun. 2013;436(4):625-31.

31. Roesch A, Fukunaga-Kalabis M, Schmidt EC, Zabierowski SE, Brafford PA, Vultur A, et al. A temporarily distinct subpopulation of slow-cycling melanoma cells is required for continuous tumor growth. Cell. 2010;141(4):583-94.

32. Chaffer CL, Marjanovic ND, Lee T, Bell G, Kleer CG, Reinhardt F, et al. Poised chromatin at the ZEB1 promoter enables breast cancer cell plasticity and enhances tumorigenicity. Cell. 2013;154(1):61-74.

33. Kurtova AV, Xiao J, Mo Q, Pazhanisamy S, Krasnow R, Lerner SP, et al. Blocking PGE2-induced tumour repopulation abrogates bladder cancer chemoresistance. Nature. 2015;517(7533):209-13.

34. Mateo F, Meca-Cortés O, Celià-Terrassa T, Fernández Y, Abasolo I, Sánchez-Cid L, et al. SPARC mediates metastatic cooperation between CSC and non-CSC prostate cancer cell subpopulations. Mol Cancer. 2014;13:237.

35. Iliopoulos D, Hirsch HA, Wang G, Struhl K. Inducible formation of breast cancer stem cells and their dynamic equilibrium with non-stem cancer cells via IL6 secretion. Proc Natl Acad Sci U S A. 2011;108(4):1397-402.

36. Kim SY, Kang JW, Song X, Kim BK, Yoo YD, Kwon YT, et al. Role of the IL-6-JAK1-STAT3-Oct-4 pathway in the conversion of non-stem cancer cells into cancer stem-like cells. Cell Signal. 2013;25(4):961-9.

37. Dean M, Fojo T, Bates S. Tumour stem cells and drug resistance. Nat Rev Cancer. 2005;5(4):275-84.

38. Emmink BL, Verheem A, Van Houdt WJ, Steller EJ, Govaert KM, Pham TV, et al. The secretome of colon cancer stem cells contains drug-metabolizing enzymes. J Proteomics. 2013;91:84-96.

39. Lagadec C, Vlashi E, Della Donna L, Meng Y, Dekmezian C, Kim K, et al. Survival and self-renewing capacity of breast cancer initiating cells during fractionated radiation treatment. Breast cancer research : BCR. 2010;12(1):R13.

40. Cho YM, Kim YS, Kang MJ, Farrar WL, Hurt EM. Long-term recovery of irradiated prostate cancer increases cancer stem cells. The Prostate. 2012;72(16):1746-56.

41. Du L, Wang H, He L, Zhang J, Ni B, Wang X, et al. CD44 is of functional importance for colorectal cancer stem cells. Clin Cancer Res. 2008;14(21):6751-60.

42. Gener P, Rafael DF, Fernández Y, Ortega JS, Arango D, Abasolo I, et al. Cancer stem cells and personalized cancer nanomedicine. Nanomedicine (Lond). 2016;11(3):307-20.

43. Chen Y, Hu Y, Zhang H, Peng C, Li S. Loss of the Alox5 gene impairs leukemia stem cells and prevents chronic myeloid leukemia. Nat Genet. 2009;41(7):783-92.

44. Chen Y, Sullivan C, Peng C, Shan Y, Hu Y, Li D, et al. A tumor suppressor function of the Msr1 gene in leukemia stem cells of chronic myeloid leukemia. Blood. 2011;118(2):390-400.

45. Chen Y, Peng C, Sullivan C, Li D, Li S. Novel therapeutic agents against cancer stem cells of chronic myeloid leukemia. Anticancer Agents Med Chem. 2010;10(2):111-5.

46. Gupta PB, Onder TT, Jiang G, Tao K, Kuperwasser C, Weinberg RA, et al. Identification of selective inhibitors of cancer stem cells by high-throughput screening. Cell. 2009;138(4):645-59.

47. Naujokat C, Steinhart R. Salinomycin as a drug for targeting human cancer stem cells. J Biomed Biotechnol. 2012;2012:950658.

48. Boehmerle W, Endres M. Salinomycin induces calpain and cytochrome c-mediated neuronal cell death. Cell Death Dis. 2011;2:e168.

49. Zhao J, Feng SS. Nanocarriers for delivery of siRNA and co-delivery of siRNA and other therapeutic agents. Nanomedicine (Lond). 2015;10(14):2199-228.

50. Kang Y, Massague J. Epithelial-mesenchymal transitions: twist in development and metastasis. Cell. 2004;118(3):277-9.

51. Ozpolat B, Sood AK, Lopez-Berestein G. Nanomedicine based approaches for the delivery of siRNA in cancer. Journal of internal medicine. 2010;267(1):44-53.

52. Agiostratidou G, Hulit J, Phillips GR, Hazan RB. Differential cadherin expression: potential markers for epithelial to mesenchymal transformation during tumor progression. Journal of mammary gland biology and neoplasia. 2007;12(2-3):127-33.

53. Kalluri R. EMT: when epithelial cells decide to become mesenchymal-like cells. The Journal of clinical investigation. 2009;119(6):1417-9.

54. Peter ME. Let-7 and miR-200 microRNAs: guardians against pluripotency and cancer progression. Cell Cycle. 2009;8(6):843-52.

55. Ceppi P, Peter ME. MicroRNAs regulate both epithelial-to-mesenchymal transition and cancer stem cells. Oncogene. 2014;33(3):269-78.

56. Thiery JP, Acloque H, Huang RY, Nieto MA. Epithelial-mesenchymal transitions in development and disease. Cell. 2009;139(5):871-90.

57. Zeisberg M, Neilson EG. Biomarkers for epithelial-mesenchymal transitions. The Journal of clinical investigation. 2009;119(6):1429-37.

58. Yang J, Weinberg RA. Epithelial-mesenchymal transition: at the crossroads of development and tumor metastasis. Developmental cell. 2008;14(6):818-29.

59. Brabletz T. EMT and MET in metastasis: where are the cancer stem cells? Cancer Cell. 2012;22(6):699-701.

60. Brabletz T, Jung A, Spaderna S, Hlubek F, Kirchner T. Opinion: migrating cancer stem cells - an integrated concept of malignant tumour progression. Nat Rev Cancer. 2005;5(9):744-9.

61. Maier HJ, Wirth T, Beug H. Epithelial-Mesenchymal Transition in Pancreatic Carcinoma. Cancers. 2010;2(4):2058-83.

62. Berx G. The E-cadherin/catenin complex: an important gatekeeper in breast cancer tumorigenesis and malignant progression. 2001.

63. Cowin P, Rowlands TM, Hatsell SJ. Cadherins and catenins in breast cancer. Current opinion in cell biology. 2005;17(5):499-508.

64. NivesPećina-Slaus. Tumor suppressor gene E-cadherin and its role in normal and malignant cells. 2003.

65. Xu J, Lamouille S, Derynck R. TGF-beta-induced epithelial to mesenchymal transition. Cell research. 2009;19(2):156-72.

66. Onder TT, Gupta PB, Mani SA, Yang J, Lander ES, Weinberg RA. Loss of E-cadherin promotes metastasis via multiple downstream transcriptional pathways. Cancer research. 2008;68(10):3645-54.

67. Thiery JP. Epithelial-mesenchymal transitions in tumour progression. Nature reviews Cancer. 2002;2(6):442-54.

68. Wong AS, Gumbiner BM. Adhesion-independent mechanism for suppression of tumor cell invasion by E-cadherin. The Journal of cell biology. 2003;161(6):1191-203.

69. Gravdal K, Halvorsen OJ, Haukaas SA, Akslen LA. A switch from E-cadherin to N-cadherin expression indicates epithelial to mesenchymal transition and is of strong and independent importance for the progress of prostate cancer. Clinical cancer research : an official journal of the American Association for Cancer Research. 2007;13(23):7003-11.

70. Clark CJ, Sage EH. A prototypic matricellular protein in the tumor microenvironment--where there's SPARC, there's fire. J Cell Biochem. 2008;104(3):721-32.

71. Robert G, Gaggioli C, Bailet O, Chavey C, Abbe P, Aberdam E, et al. SPARC represses E-cadherin and induces mesenchymal transition during melanoma development. Cancer Res. 2006;66(15):7516-23.

72. Weaver MS, Workman G, Sage EH. The copper binding domain of SPARC mediates cell survival in vitro via interaction with integrin beta1 and activation of integrin-linked kinase. J Biol Chem. 2008;283(33):22826-37.

73. Shi Q, Bao S, Song L, Wu Q, Bigner DD, Hjelmeland AB, et al. Targeting SPARC expression decreases glioma cellular survival and invasion associated with reduced activities of FAK and ILK kinases. Oncogene. 2007;26(28):4084-94.

74. Barker TH, Baneyx G, Cardó-Vila M, Workman GA, Weaver M, Menon PM, et al. SPARC regulates extracellular matrix organization through its modulation of integrin-linked kinase activity. J Biol Chem. 2005;280(43):36483-93.

75. Bradshaw AD, Sage EH. SPARC, a matricellular protein that functions in cellular differentiation and tissue response to injury. J Clin Invest. 2001;107(9):1049-54.

76. Zou W. Immunosuppressive networks in the tumour environment and their therapeutic relevance. Nat Rev Cancer. 2005;5(4):263-74.

77. Moustakas A, Souchelnytskyi S, Heldin CH. Smad regulation in TGF-beta signal transduction. J Cell Sci. 2001;114(Pt 24):4359-69.

78. Katsuno Y, Lamouille S, Derynck R. TGF-β signaling and epithelial-mesenchymal transition in cancer progression. Curr Opin Oncol. 2013;25(1):76-84.

79. Smad Signal Transduction: Smads in Proliferation, Differentiation and Disease: Springer; 2006. 473 p.

80. Vincent T, Neve EP, Johnson JR, Kukalev A, Rojo F, Albanell J, et al. A SNAIL1-SMAD3/4 transcriptional repressor complex promotes TGF-beta mediated epithelial-mesenchymal transition. Nat Cell Biol. 2009;11(8):943-50.

81. Xue G, Restuccia DF, Lan Q, Hynx D, Dirnhofer S, Hess D, et al. Akt/PKB-mediated phosphorylation of Twist1 promotes tumor metastasis via mediating cross-talk between PI3K/Akt and TGF-β signaling axes. Cancer Discov. 2012;2(3):248-59.

82. Parvani JG, Galliher-Beckley AJ, Schiemann BJ, Schiemann WP. Targeted inactivation of β1 integrin induces β3 integrin switching, which drives breast cancer metastasis by TGF-β. Mol Biol Cell. 2013;24(21):3449-59.

83. Parsons JT, Horwitz AR, Schwartz MA. Cell adhesion: integrating cytoskeletal dynamics and cellular tension. Nat Rev Mol Cell Biol. 2010;11(9):633-43.

84. Arboleda MJ. Overexpression of AKT2/Protein Kinase Bb Leads to Up-Regulation of b1 Integrins, Increased Invasion, and Metastasis of Human Breast and Ovarian Cancer Cells. 2003.

85. Virtakoivu R, Pellinen T, Rantala JK, Perälä M, Ivaska J. Distinct roles of AKT isoforms in regulating β1-integrin activity, migration, and invasion in prostate cancer. Mol Biol Cell. 2012;23(17):3357-69.

86. Régent M, Planus E, Bouin AP, Bouvard D, Brunner M, Faurobert E, et al. Specificities of β1 integrin signaling in the control of cell adhesion and adhesive strength. Eur J Cell Biol. 2011;90(2-3):261-9.

87. Vassos N, Rau T, Merkel S, Feiersinger F, Geppert CI, Stürzl M, et al. Prognostic value of β1 integrin expression in colorectal liver metastases. Int J Clin Exp Pathol. 2013;7(1):288-300.

88. Moreno-Bueno G, Cubillo E, Sarrio D, Peinado H, Rodriguez-Pinilla SM, Villa S, et al. Genetic profiling of epithelial cells expressing E-cadherin repressors reveals a distinct role for Snail, Slug, and E47 factors in epithelial-mesenchymal transition. Cancer research. 2006;66(19):9543-56.

89. Vesuna F, van Diest P, Chen JH, Raman V. Twist is a transcriptional repressor of E-cadherin gene expression in breast cancer. Biochemical and biophysical research communications. 2008;367(2):235-41.

90. Yang J, Mani SA, Donaher JL, Ramaswamy S, Itzykson RA, Come C, et al. Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. Cell. 2004;117(7):927-39.

91. Wang X, Ling MT, Guan XY, Tsao SW, Cheung HW, Lee DT, et al. Identification of a novel function of TWIST, a bHLH protein, in the development of acquired taxol resistance in human cancer cells. Oncogene. 2004;23(2):474-82.

92. Cheng GZ, Chan J, Wang Q, Zhang W, Sun CD, Wang LH. Twist transcriptionally up-regulates AKT2 in breast cancer cells leading to increased migration, invasion, and resistance to paclitaxel. Cancer research. 2007;67(5):1979-87.

93. Cheng GZ, Zhang W, Wang LH. Regulation of cancer cell survival, migration, and invasion by Twist: AKT2 comes to interplay. Cancer research. 2008;68(4):957-60.

94. Stambolic V, Woodgett JR. Functional distinctions of protein kinase B/Akt isoforms defined by their influence on cell migration. Trends in cell biology. 2006;16(9):461-6.

95. Gonzalez E. The Akt kinases: isoform specificity in metabolism and cancer. 2009.

96. Fresno Vara JA, Casado E, de Castro J, Cejas P, Belda-Iniesta C, Gonzalez-Baron M. PI3K/Akt signalling pathway and cancer. Cancer treatment reviews. 2004;30(2):193-204.

97. Irie HY, Pearline RV, Grueneberg D, Hsia M, Ravichandran P, Kothari N, et al. Distinct roles of Akt1 and Akt2 in regulating cell migration and epithelial-mesenchymal transition. The Journal of cell biology. 2005;171(6):1023-34.

98. Hanada M, Feng J, Hemmings BA. Structure, regulation and function of PKB/AKT—a major therapeutic target. Biochimica et Biophysica Acta (BBA) - Proteins & Proteomics. 2004;1697(1-2):3-16.

99. Chong ZZ, Li F, Maiese K. Activating Akt and the brain's resources to drive cellular survival and prevent inflammatory injury. Histol Histopathol. 2005;20(1):299-315.

100. Grille SJ. Invasiveness of Squamous Cell Carcinoma Lines Transition and Promotes Enhanced Motility and The Protein Kinase Akt Induces Epithelial Mesenchymal. 2003.

101. Tschopp O, Yang ZZ, Brodbeck D, Dummler BA, Hemmings-Mieszczak M, Watanabe T, et al. Essential role of protein kinase B gamma (PKB gamma/Akt3) in postnatal brain development but not in glucose homeostasis. Development. 2005;132(13):2943-54.

102. Linnerth-Petrik NM, Santry LA, Petrik JJ, Wootton SK. Opposing Functions of Akt Isoforms in Lung Tumor Initiation and Progression. PLoS One. 2014;9(4):e94595.

103. Sasaki T, Nakashiro K, Tanaka H, Azuma K, Goda H, Hara S, et al. Knockdown of Akt isoforms by RNA silencing suppresses the growth of human prostate cancer cells in

vitro and in vivo. Biochemical and biophysical research communications. 2010;399(1):79-83.

104. Attoub S, Arafat K, Hammadi NK, Mester J, Gaben A-M. Akt2 knock-down reveals its contribution to human lung cancer cell proliferation, growth, motility, invasion and endothelial cell tube formation. Scientific Reports, Published online: 3 August 2015; | doi:101038/srep12759. 2015;5(12759):1-14.

105. Altomare DA, Testa JR. Perturbations of the AKT signaling pathway in human cancer. Oncogene. 2005;24(50):7455-64.

106. Vestey SB, Sen C, Calder CJ, Perks CM, Pignatelli M, Winters ZE. Activated Akt expression in breast cancer: correlation with p53, Hdm2 and patient outcome. European journal of cancer. 2005;41(7):1017-25.

107. Lee CS, Kim YJ, Jang ER, Myung SC, Kim W. Akt inhibitor enhances apoptotic effect of carboplatin on human epithelial ovarian carcinoma cell lines. European journal of pharmacology. 2010;632(1-3):7-13.

108. Wang J, Wan W, Sun R, Liu Y, Sun X, Ma D, et al. Reduction of Akt2 expression inhibits chemotaxis signal transduction in human breast cancer cells. Cell Signal. 2008;20(6):1025-34.

109. Cheng GZ, Zhang WZ, Sun M, Wang Q, Coppola D, Mansour M, et al. Twist is transcriptionally induced by activation of STAT3 and mediates STAT3 oncogenic function. The Journal of biological chemistry. 2008;283(21):14665-73.

110. Zhang L, Sun S, Zhou J, Liu J, Lv JH, Yu XQ, et al. Knockdown of Akt1 promotes Akt2 upregulation and resistance to oxidative-stress-induced apoptosis through control of multiple signaling pathways. Antioxid Redox Signal. 2011;15(1):1-17.

111. Kanasty R, Dorkin JR, Vegas A, Anderson D. Delivery materials for siRNA therapeutics. Nature Materials. 2013;12:967-77.

112. Mohr SE, Smith JA, Shamu CE, Neumüller RA, Perrimon N. RNAi screening comes of age: improved techniques and complementary approaches. Nat Rev Mol Cell Biol. 2014;15(9):591-600.

113. Kapoor M, Burgess DJ, Patil SD. Physicochemical characterization techniques for lipid based delivery systems for siRNA. Int J Pharm. 2012;427(1):35-57.

114. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004;116(2):281-97.

115. Pillai RS, Bhattacharyya SN, Filipowicz W. Repression of protein synthesis by miRNAs: how many mechanisms? Trends Cell Biol. 2007;17(3):118-26.

116. Alvarez JP, Pekker I, Goldshmidt A, Blum E, Amsellem Z, Eshed Y. Endogenous and synthetic microRNAs stimulate simultaneous, efficient, and localized regulation of multiple targets in diverse species. Plant Cell. 2006;18(5):1134-51.

117. Jackson AL, Linsley PS. Recognizing and avoiding siRNA off-target effects for target identification and therapeutic application. Nat Rev Drug Discov. 2010;9(1):57-67.

118. Whitehead KA, Langer R, Anderson DG. Knocking down barriers: advances in siRNA delivery. Nat Rev Drug Discov. 2009;8(2):129-38.

119. Singh SK, Hajeri P. siRNAs: their potential as therapeutic agents – Part II. Methods of delivery. Drug Discovery Today. 2009;14(17-18):859-65.

120. Tokatlian T, Segura T. siRNA applications in nanomedicine. Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology. 2010;2(3):305-15.

121. Friedman JM, Jones PA. MicroRNAs: critical mediators of differentiation, development and disease. Swiss Med Wkly. 2009;139(33-34):466-72.

122. Deng Y, Wang CC, Choy KW, Du Q, Chen J, Wang Q, et al. Therapeutic potentials of gene silencing by RNA interference: Principles, challenges, and new strategies. Gene. 2014.

123. Carthew RW, Sontheimer EJ. Origins and Mechanisms of miRNAs and siRNAs. Cell. 2009;136(4):642-55.

124. Iliopoulos D, Polytarchou C, Hatziapostolou M, Kottakis F, Maroulakou IG, Struhl K, et al. MicroRNAs differentially regulated by Akt isoforms control EMT and stem cell renewal in cancer cells. Sci Signal. 2009;2(92):ra62.

125. Valastyan S, Weinberg RA. Roles for microRNAs in the regulation of cell adhesion molecules. J Cell Sci. 2011;124(Pt 7):999-1006.

126. Park SM, Gaur AB, Lengyel E, Peter ME. The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2. Genes Dev. 2008;22(7):894-907.

127. Schmitt MJ, Margue C, Behrmann I, Kreis S. MiRNA-29: a microRNA family with tumor-suppressing and immune-modulating properties. Curr Mol Med. 2013;13(4):572-85.

128. Cheng JQ. AKT2, a putative oncogene encoding a member of a subfamily of protein-serine/threonine kinases, is amplified in human ovarian carcinomas. 1992.

129. CHENG JQ. Amplification ofAKT2 in human pancreatic cancer cells and inhibition ofAKT2 expression and tumorigenicity by antisense RNA. 1996.

130. Pu P, Kang C, Li J, Jiang H, Cheng J. The effects of antisense AKT2 RNA on the inhibition of malignant glioma cell growth in vitro and in vivo. Journal of neuro-oncology. 2006;76(1):1-11.

131. Rajput S, Puvvada N, Kumar BN, Sarkar S, Konar S, Bharti R, et al. Overcoming Akt Induced Therapeutic Resistance in Breast Cancer through siRNA and Thymoquinone Encapsulated Multilamellar Gold Niosomes. Mol Pharm. 2015;12(12):4214-25.

CHAPTER 2

State of Art – Nanotechnology for Gene Delivery

The information presented in this chapter was partially published in the following publications:

1) P Gener, D Rafael, S Schwartz Jr., F Andrade, The Emerging Role of Nanomedicine in the Advances of Oncological Treatment in *Nanoparticles in the Life Sciences and Biomedicine*, Salette Reis and Ana Rute Neves (Eds.), Pan Stanford Publishing Pte Ltd, 2017, *in press*.
2) D Rafael, M Videira, M Oliva, D Ferreira, B Sarmento, F Andrade, Amphiphilic Polymers in Drug Delivery in *Encyclopedia of Biomedical Polymers and Polymeric Biomaterials*, Munmaya Mishra (Ed.), CRC Press, 2015, ISBN 9781439898796.
3) D Rafael, F Andrade, A Arranja, AS Luís, M Videira, Lipoplexes and Polyplexes: Gene Delivery Applications in *Encyclopedia of Biomedical Polymers and Polymeric Biomaterials*, Munmaya Mishra (Ed.), CRC Press, 2015, ISBN 9781439898796.
4) M Videira, A Arranja, D Rafael, R Gaspar, Preclinical development of siRNA therapeutics: towards the match between fundamental science and engineered systems, Nanomedicine, 10 (4):689–702, 2014.
5) F Andrade, D Rafael, M Videira, D Ferreira, A Sosnik, B Sarmento, Nanotechnology and pulmonary delivery to overcome resistance in infectious diseases, Advanced Drug Delivery Reviews, 65 (13–14):1816–1827, 2013.
6) J Viola, D Rafael, E Wagner, R Besch, M Ogris. “Gene Therapy for Advanced Melanoma: Selective Targeting and Therapeutic Nucleic Acids”, in Journal of Drug Delivery, Volume 2013 (2013), Article ID 897348.

Table of contents

2.1. The Problematic Choice of a Vector for Gene Therapy

Gene therapy has caught the attention of several researchers as an opportunity to treat both acquired and innate diseases. Considerable attention was concentrated in biology-driven therapies since a four-year old girl with severe combined immunodeficiency (SCID) caused by adenosine deaminase deficiency was treated using a virus based DNA delivery system in the first gene therapy clinical trial performed in the 1990s (1, 2). Delivery and subsequent expression of an exogenous oligonucleotide encoding for a missing or defective gene or to achieve the silence of a particular gene of interest, such as an oncogene are different approaches that bring new alternatives and effective resolutions for the treatment of several diseases, including hematological, immunological, ocular, and neurodegenerative diseases, and especially for the so feared cancer (3).

The use of gene therapy in cancer seems to be the utmost alternative to overcome the well-known drug-related secondary adverse effects, chemoresistance and problems associated with the radiotherapy. However, *in vivo* delivery of OGN has precluding the clinical utilization of gene-based therapies mainly due to its vulnerability to enzymatic degradation, poor cellular uptake and rapid renal clearance. Therefore, the great challenge for the successful clinical applications of gene therapy demands the emergence of new tools to enable the negatively charged OGN to be effectively delivered into target cells by crossing the hydrophobic cell membrane and reach the cytoplasm and/or nucleus (4-6). The case of the patient that developed leukemia after successful treatment of SCID using gene therapy probably own to an insertional mutagenesis event associated with the retrovirus vector used, slowed down the euphoria observed with the positive and encouraging results from the first clinical trials and imposed an reassessment of the strategies used for gene transfer (7). However, during the last decades, some positive results regarding restoration of vision in blind patients, eradication of blood cancers or correction of hemoglobinopathies, especially in pediatric patients, gave hope to the researchers to continuing the development and clinical evaluation of gene-based therapies (about 2000 clinical studies up to now) (3, 4). Still, until now only Glybera® (alipogene tiparvovec using an adeno-associated virus as vector) was granted with marketing authorization by European Commission in late 2012 to treat lipoprotein lipase deficiency (8).

The vectors used for gene delivery can be divided in two main groups: viral and non-viral vectors. Nowadays, viral vectors are still considered the most efficient, being the most commonly used for gene transfer in both pre-clinical and clinical research (3). However, the well-known drawbacks related with viral-based vectors such as their immunogenicity, mutagenesis, carcinogenesis, limited cargo loading, and time consuming/high cost procedures, boosted the development of safer vehicles using a wide range of lipids and

polymers (9, 10). This explains the enormous outbreak of the non-viral vectors in the last years (9, 11-14).

Nevertheless, several limitations were also identified in some of the different non-viral vectors, such as low specificity, cellular toxicity, low biodegradability, rapid blood-circulation clearance, or limited transfection efficiencies (5, 15). Additionally, the steps involved in the gene delivery mediated by these vectors faces biological barriers as described in **Figure 2.1.** As endocytosis represents the leading pathway for nanoparticles internalization, endosomal escape is probably one of the most important steps related with the transfection efficiency. It is well-known that after endocytosis, nanoparticles remain sequestered in vesicles (endosomes) that release the genetic material upon vector degradation; however, if naked OGN are not released from the endosome, their degradation will occurs.

Therefore, for effective gene delivery, the vectors must contain compounds presenting endosomal membrane disruptive properties, which allows the genetic material to be release into the cytosol, continuing its journey (16-18). The mechanism inherent to endosomal disruption and the consequent nucleic acids release into the cytoplasm is the so-called “proton-sponge effect”. This phenomenon is defined as the vectors ability to buffer the endosomal pH, inducing endosomes osmotic swelling due to the excessive proton and chloride accumulation leading to a secondary water movement, and consequent disruption (16, 19).

Regarding siRNA delivery, upon endosomes disruption the siRNA sequences are available in the cytosol to initiate its therapeutic RNAi mechanism. The same does not apply to DNA, which still faces additional barriers before being able to exert its effect in the nucleus **(Figure 2.1)** (17).

The ideal gene delivery system should be efficient, stable, cost effective, and capable to avoid rapid hepatic or renal clearance. Safety issues such as being biocompatible, biodegradable and non-immunogenic are also critical. As a pharmaceutical system, their functionalization and sustained-release profiles are known advantages among these systems. However, it is always necessary an appropriate balance between protection and release of the genetic material in order to ensure its biological functionality (5, 20).

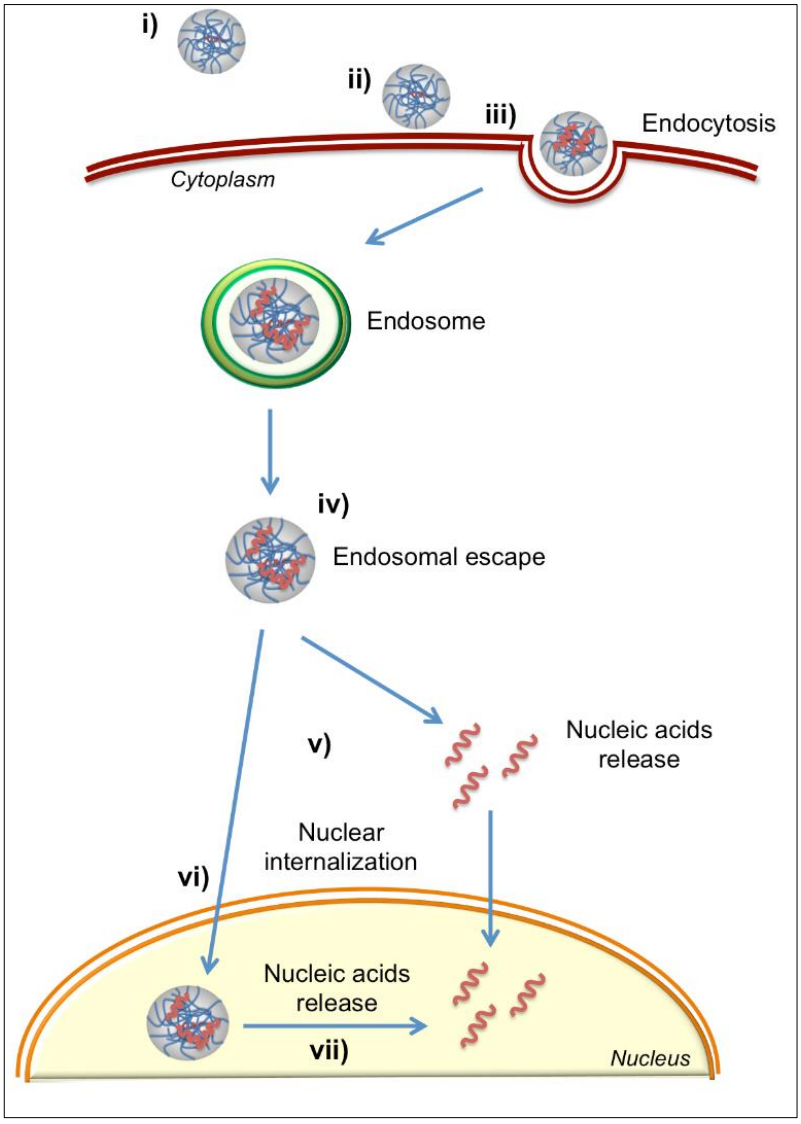


Figure 2. 1. *In vitro* barriers for non-viral vectors based gene delivery. i) the nucleic acids complexation, that is dependent on the charge of the lipid or polymer, ii) the binding of lipoplexes and polyplexes to the cellular membrane that, except in the presence of specific targeting ligands, is usually nonspecific, iii) the particles internalization, that usually occurs via one of the five major endocytic pathways (phagocytosis, macropinocytosis, clathrin-mediated and caveolae-mediated or independent endocytosis), iv) the endosomal escape, one of the more critical phenomenon since is dependent on the occurrence of the proton-sponge effect, v) the cytoplasmic transportation, vi) the entry to the nucleus, and vii) the decomplexation of DNA-vector within the nucleus and integrate the host genome.

2.2. Non-viral Gene Delivery Systems

Different systems based on nanotechnology have been developed and proposed to reach the perfect gene delivery system (9, 10). **Figure 2.2** summarizes the different types of nanoparticles commonly used as non-viral gene delivery systems.

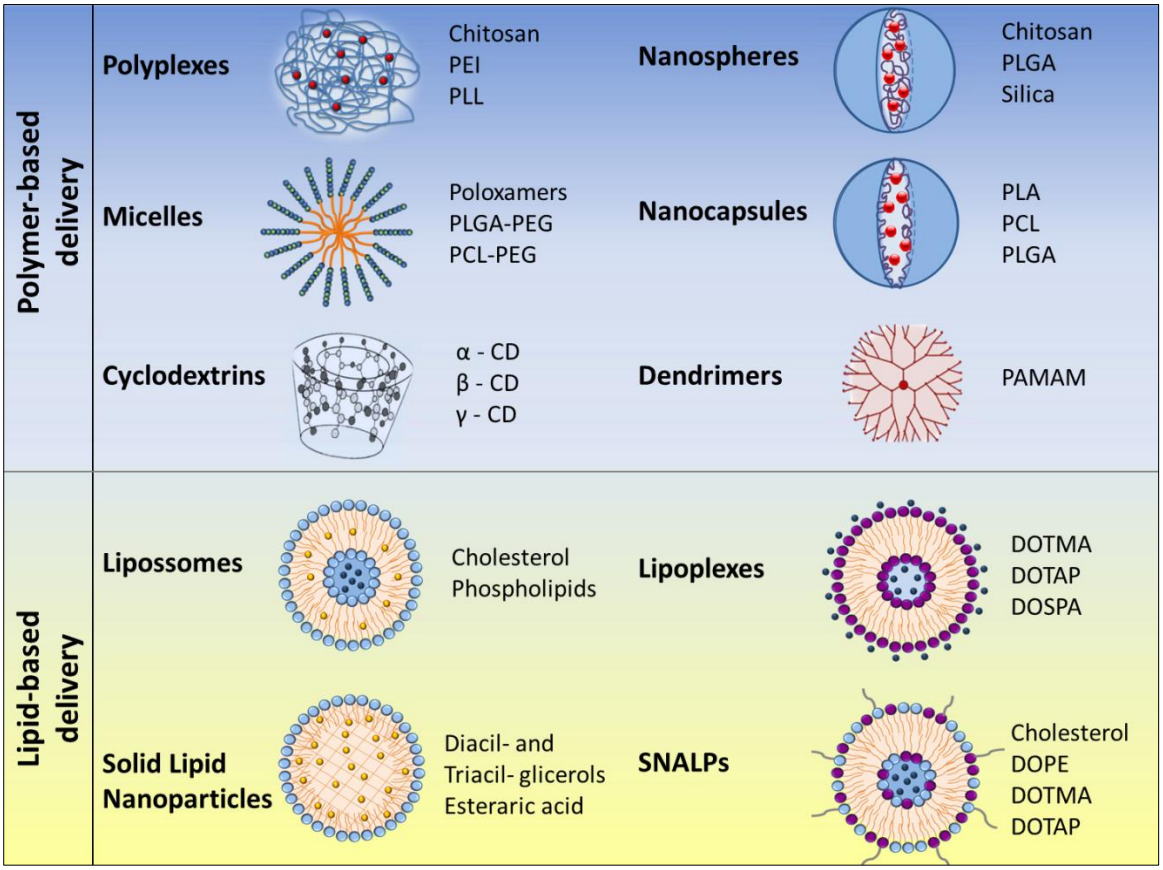


Figure 2. 2. Different types of nanotechnology-based systems for gene delivery. The main polymer and lipid-based systems that can be used for gene delivery are schematized, as well as the components commonly used in its production. CD: cyclodextrin; DOPE: 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphoethanolamine; DOSPA: 2,3-dioleyloxy-N-[2(sperminecarboxamido) ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate; DOTAP: 1,2-dioleoyl-3-trimethylammonium-propane; DOTMA: 1,2-di-O-octadecenyl-3-trimethylammonium propane; PAMAM: polyamidoamine; PCL: poly(ε-caprolactone); PEI: polyethylenimine; PLA: poly(d,l-lactide); PLGA: poly(d,l-lactide-co-glycolide); PLL: poly(L-lysine); SNALPs: stable nucleic acid lipid particles.

During the design of non-viral vectors, characteristics such as particle size, surface charge and presence of biological moieties should be finely tuned in order to improve their biological behavior and activity like blood circulation half-life, transfection activity and intracellular trafficking (21, 22). For this reason, nanoparticles intended for gene delivery usually possess in its composition cationic lipids or polymers that interact with the negatively charged nucleic acids by electrostatic interactions and condense into complexes (lipoplexes or polyplexes). The resultant polycation-based gene delivery systems net charge dictates the interaction with serum proteins and cell membrane surface (23-26). Usually, a positive charge promotes the interaction with negatively charged cell membrane, but also undesired associations with serum proteins. Therefore, to overcome these safety issues, lipo- and polyplexes are usually modified or conjugated with another lipids and/or polymers, affecting the nucleic acids condensation, nanoparticle serum stability and cell surface activity (24).

The main advantages and disadvantages related with nanoparticles as gene delivery systems are summarized in **Figure 2.3.**

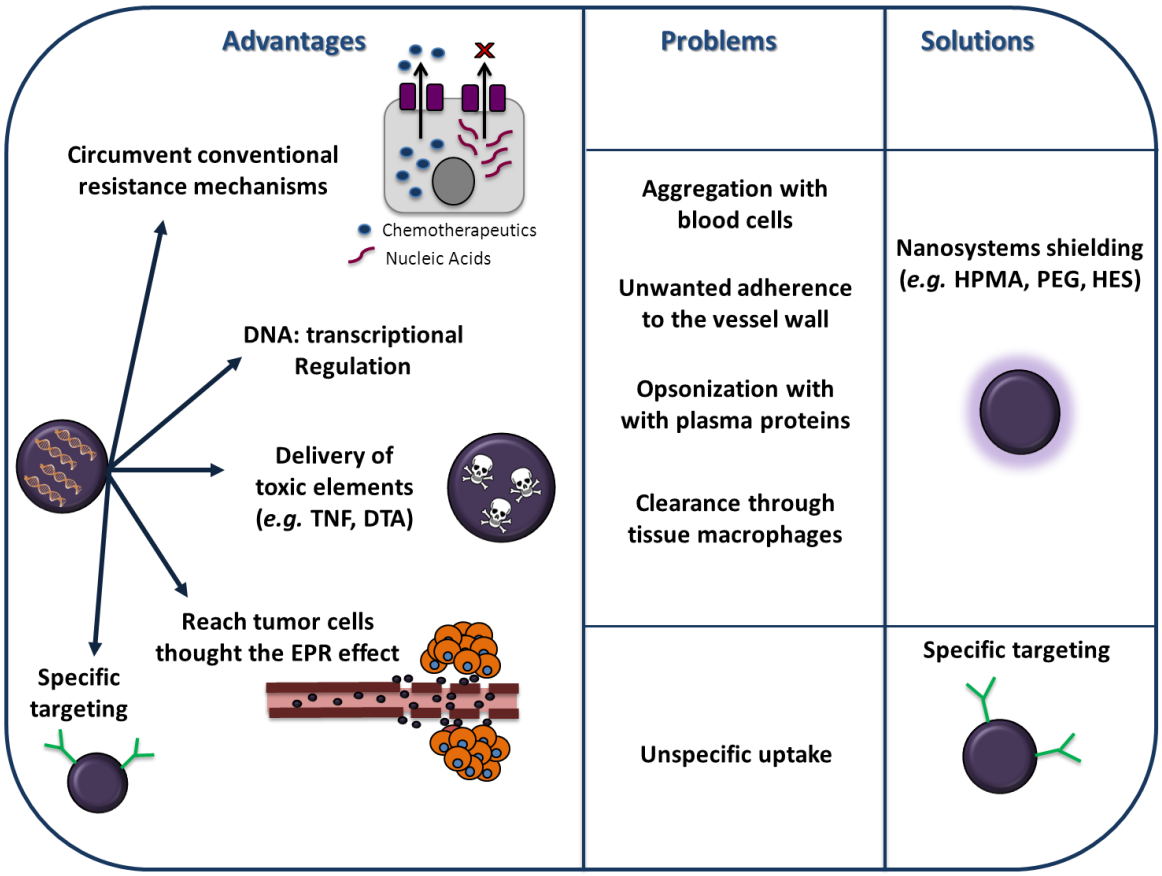


Figure 2. 3. Advantages and limitations in nucleic acid nanosystems delivery. Particular advantages of nucleic acid therapies are (1) the ability to include tissue specific targeting (or transcriptional targeting) and (2) the possibility to systemically deliver genes encoding for proteins with toxic properties. Moreover, as macromolecules, nucleic acids can overcome resistance mechanisms such as that supported by P-gp. However, nucleic acids are vulnerable in blood circulation, and hence they must be protected against enzyme degradation and condensed in the form of polyplexes. Physiological barriers, such as reticulo-endothelial system, still present a threat for nanosystems, and these must be armed against possible interactions with blood cells that can result in opsonization or undesired blood vessel adhesion. Decoration of nanocarriers with PEG or HPMA can provide shielding effect, while decoration with ligands that can bind receptors overexpressed in tumors can assist in cellular targeting and internalization. TNF: tumor necrosis factor; DTA: diphteria toxin A; HPMA: N-(2-hydroxypropyl)methacrylamide; PEG: polyethylene glycol; HES: hydroxyethyl starch.

Oncology is the field that has most benefiting from the application of nanotechnology to drug delivery, including gene therapy. In addition, to condensate and prevent the degradation of genetic material, taking advantage of their lower particle size and the unique properties of tumor vasculature, nanotechnology-based vectors easily reach extravascular spaces becoming concentrated preferentially inside tumor tissues. Tumor vasculature

presents 600-800 nm gaps between adjacent endothelial cells as a result of increased levels of vascular mediators such as vascular endothelial growth factor (VEGF), nitric oxide, prostaglandins and bradykinins. This characteristic leaky architecture of tumor vessels, in combination with a poor lymphatic drainage is responsible for the enhanced permeability and retention (EPR) effect, the drug/gene-loaded nanoparticles natural tendency to accumulate into tumor tissue is the base of the passive targeting strategy (27-31). Of note, ubiquitously targeting cells within a tumor is not always feasible because some drugs cannot diffuse efficiently. The random nature of the passive targeting makes difficult to control drugs' pharmacological effectiveness and this may induce MDR events. In order to increase the specificity of the vectors to tumor cells, beyond this passive targeting, chemical modification of the particles surface with specific moieties like antibody fragments or ligands that recognize and interact with membrane receptors or antigens specifically overexpressed in the target cancer cell surfaces represents a suitable approach (22, 27, 32, 33). As reffered in **Chapter 1**, special attention has been given to the targeting of CSC in order to provide new innovative treatments for patients with advanced cancer and reduce tumor relapse **(Figure 2.4)**. An additional advantage is related with the fact that some drugs will benefit from entering inside tumor cells by receptor-mediated endocytosis, providing higher concentration levels of the active agent compounds within the tumor cell. These second generation of vectors might be especially useful in tumors that do not exhibit EPR effect or cases where the permeability of the vessels is not compromised (i.e.small metastases).

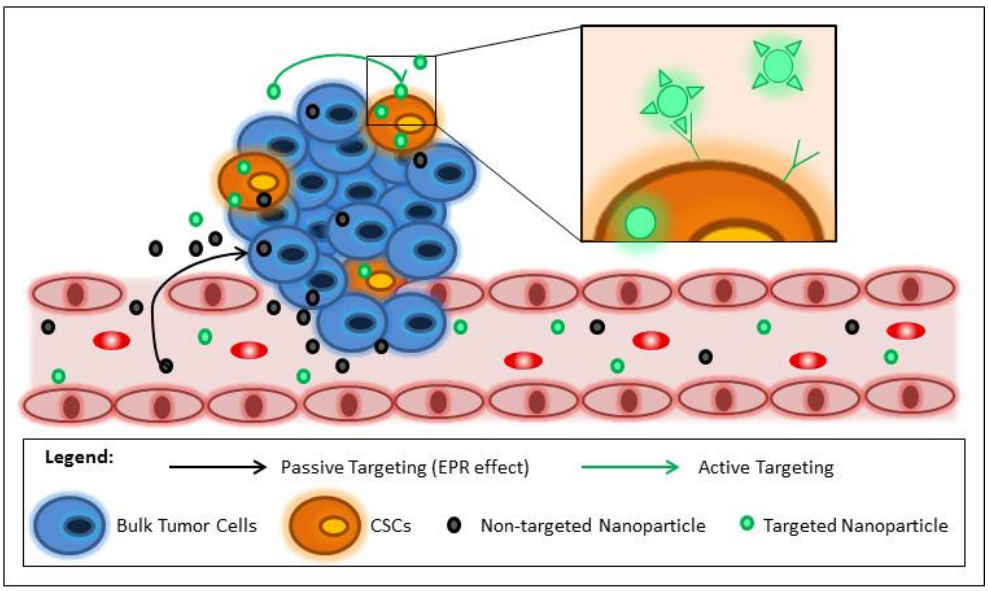


Figure 2. 4. Schematic representation of the mechanisms of nanoDDS for CSC targeting. EPR effect versus active targeting. CSC: cancer stem cells; DDS: drug delivery system; EPR: enhanced permeability and retention.

2.2.1. Polymer-based Delivery Systems (Polyplexes)

2.2.1.1. Cationic polymers

Biodegradable polymeric nanoparticles have been highly proposed as gene delivery systems, not only due to higher complexation capacity, but also to the improved endosomal escape properties, resulting in enhanced transfection efficiencies (22, 34-38). Due to an easiest interaction with the negatively charged OGN and cellular membranes, greater attention is given to the cationic charged polymers (39-41) since its first proposal in 1987 by Wu GY & Wu CH (42).

The most commonly used polycations are synthetic cationic polymers such as chitosan (CS) and its derivatives, branched or linear PEI and its derivatives, polyamidoamine (PAMAM), poly(b-amino ester)s (PAEs), poly(2-aminoethyl ethylene phosphate) (PPEEA), and poly(2-(N,N-dimethylamino)ethyl methacrylate) (PDMAEMA) (15, 43, 44). Cationic peptides and polypeptides such as the ones based on histidine (45, 46), protamine (47, 48), lysine (poly(L-lysine) (PLL)) (49, 50) are also been proposed as polymeric non-viral vectors (51-54). CS is a natural cationic mucopolysaccharide obtained as a raw material from chitin deacetylation that has been shown to be biocompatible, non-inflammatory, non-toxic and biodegradable. This polymer unique structural feature is associated to the presence of the primary amine at the C-2 position of the glucosamine residues that promotes a high positive charge responsible for the polyelectrolyte complexes formation with negatively charged nucleotides (55). The properties of CS mainly for siRNA and DNA-mediated gene therapy depend on the molecular weight (MW) and degree of deacetylation (DD) as it affects the charge density, solubility, hydrophobicity and its ability to interact electrostatically with polyanions (56, 57). A high DD (over 80%) has been identified as the most influential factor in efficient siRNA-mediated knockdown. Regarding the MW, Liu X, *et al* (2007) reported that CS molecules 5–10 times the length of the siRNA, could form stable nanoparticles resulting in high gene silencing efficiency in H1299 human lung carcinoma cells (58). The molar ratio (N/P ratio) between the positive charges of the polymer (amine groups) and the negative charges of the OGN (phosphate groups) also influences the polyplexes gene knockdown efficiency, partially due to the increase in nanoparticle stability specially observed at high ratio values. For example, polyplexes prepared with a 170 kDa (DD 84%) CS were only able to promote Green Fluorescent Protein (GFP) knockdown in H1299 human lung carcinoma at higher N/P ratios (50 and 150) (59). Despite its well-established advantages, CS has a limited buffering capacity when compared with PEI (16, 18). Therefore numerous studies are centered in CS modifications or conjugation with lipids or other polymers in order to improve nucleic acid transfection efficiency. Conjugation with PEI

(60, 61), modification with urocanic acid (62), addition of poly(propyl acrylic acid) (63), introduction of imidazole moieties (64) and CS grafting with stearic acids (65) are few examples of studies that have been performed aiming to improve the buffering and transfection properties of CS.

PEI and its derivatives, are a type of high cationic charge density polymers, presenting branched or linear forms, available in a broad range of MW (34, 66). PEI-based particles are well-known and well characterized non-viral vectors for efficient delivery of nucleic acids including DNA and RNA both *in vitro* and *in vivo* (22, 35, 67). The high transfection efficiencies of PEI-based vectors has been mainly related to their substantial buffering capability at the endosomal pH (from pH 6.0–6.5 in early endosomes to pH 4.5–5.5 in late endosomes and lysosomes (68)) and ability to promote the endosome osmotic swelling and rupture, releasing the genetic material into the cytoplasm, thus avoiding its traffic and degradation in the lysosomal environment (22, 35, 36, 38, 69). Additionally, PEI protonable amine groups provide a powerful electrostatic capacity, which can promote strong interactions with the negatively charged nucleic acids molecules, explaining the ability of this polymer to effectively condense nucleic acids into nanoscale entities, protecting them from degradation. Moreover, PEI cationic surface charge has also a crucial role in the binding of polyplexes with cell membrane and consequent internalization (38, 69). Unfortunately, the formed positively charged polyplexes usually become unstable, showing a tendency to aggregate throughout the incubation time, depending on the surface charge and ionic strength of the medium (69, 70). Furthermore, this high cationic charge density is also responsible for PEI well-known toxicity and side effects such as membrane damage, interaction with blood cells and activation of the complement system (38, 71).

As with other polymers, key factors such as the linear versus branched structure, the branching degree and the MW are likely to affect the final PEI-based polyplexes efficiency and cytotoxicity profile (71-75). In fact, several studies demonstrate that linear PEI presents lower cytotoxicity compared to the branched ones with similar MW. However, a decreased condensation capacity of OGN due to lower amount of primary amines and lower transfection efficiencies are consequently observed (71, 72, 74, 76). Concerning the MW; high PEI MW, present increased buffering capacity and consequently high transfection efficiencies due to a higher content of primary, secondary and tertiary amines. Nevertheless, higher MW values increase cytotoxicity due to polymer precipitation and further adherence to the outer cell membrane, inducing cell necrosis (19, 71, 72, 77). The N/P between the nitrogen groups presented in the polymer and the phosphorous atom content in the nucleic acids, also proved to be a factor affecting significantly the polyplexes' efficiency. In general, complexes formed at low molar ratio in the range of 2-5 tend to

aggregate due to hydrophobic interactions. In contrast, higher N/P ratios reduce aggregation as a result of electrostatic repulsion of the higher positive surface charge of the complexes (70, 78).

The demand for non-viral vector-based solutions in the gene therapy field prompted an extensively research towards the development of PEI derivatives (34, 66). Grafting PEI with poly(ethylene glycol) (PEG), although rendering polyplexes with lower transfection efficiencies, is one of the most commonly used strategies to reduce cytotoxicity, avoid aggregation and decrease nonspecific interactions (19, 79). Alternatively, grafting with lipophilic chains such as cholesterol, propionic, succinic or palmitic acid, alkyl chains, and fatty acids demonstrated to improve PEI-based vectors characteristics such as polyplex stabilization or cell membrane interaction (19, 75, 80, 81). For example, Oskuee R, *et al.* (2010) proposed a novel carrier for siRNA delivery based on the carboxyalkylation of a branched PEI molecule. This chemical modification reduced the particles positive surface charge leading to a more hydrophobic polyplex without limiting its buffering capacity. *In vitro* results demonstrated that carboxyalkylation strongly reduced cytotoxicity of PEI and improved silencing efficacy of the delivered siRNA (19). Another example is the hydroxyl-modified PEI prepared by Dong X, *et al.* (2013) that also show lower cytotoxicity and better transfection efficiency than the unmodified PEI. Additionally, this PEI derivative also presented a greater serum-resistance capacity (82).

2.2.1.2. Amphiphilic polymers

Amphiphilic copolymers are heterogeneous polymers composed by both hydrophilic and hydrophobic units disposed in sequential or grafted blocks (generally di- and triblock-copolymers) **(Figure 2.5)**.

Varying the type or the chain length of the different units it is possible to modulate the polymer properties and its suitability for both industrial and pharmaceutical applications (83, 84). Regarding the last one, they have been used as excipients for a long time as emulsifiers, wetting, thickening and stabilizing agents of suspensions and colloidal dispersions or gel forming agents; however, they gained an increased interest in the last decades in the development of new drug delivery systems (DDS) driven by the progresses seen in the pharmaceutical sciences and nanomedicine field (84, 85). Applications in nucleic acid delivery systems are particular exciting, once they demonstrate to improve biological activity while protecting nucleic acids from degradation and clearance by serum nucleases and immune system components (84, 85).

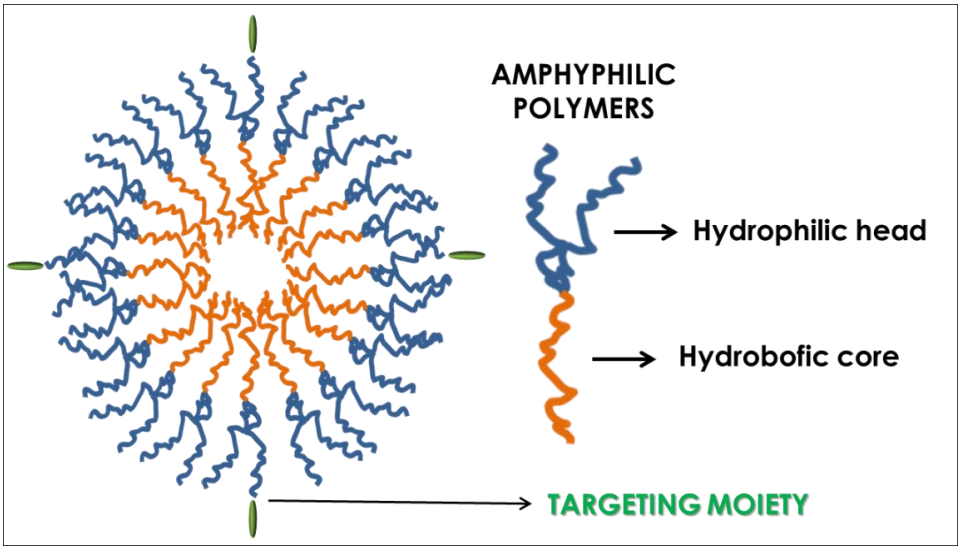


Figure 2. 5. Diagram of an amphiphilic polymers-based micelle.

The main characteristics of amphiphilic copolymers and copolymer-based structures that make them suitable for human administration are water-solubility, biodegradability, biocompatibility and low immunogenicity (**Figure 2.6)**.

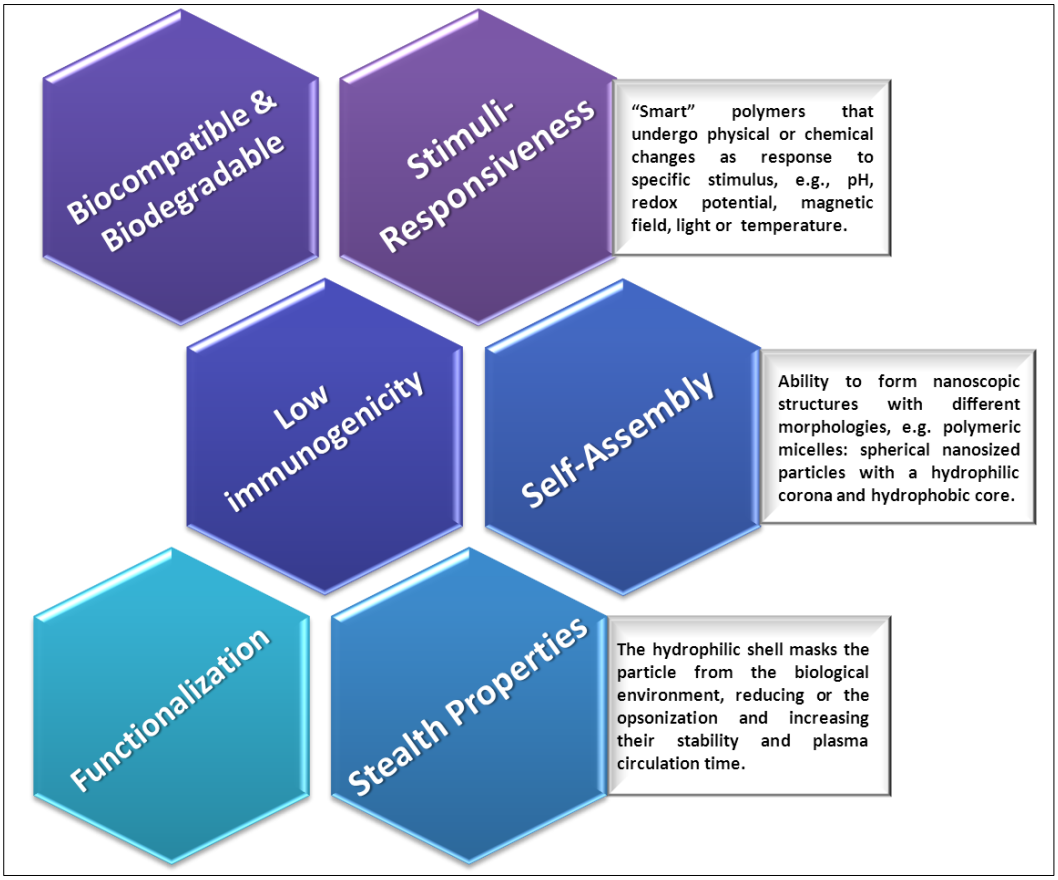


Figure 2. 6. The main features of amphiphilic copolymers and their based structures.

Among the different structures available, the most extensively researched for the production of DDS are composed of PEG as hydrophilic block and a variety of hydrophobic blocks, namely: (i) polypropylene oxice (PPO); (ii) poly(ester)s like poly(ε-caprolactone) (PCL) or poly(d,l-lactide-co-glycolide) (PLGA); (iii) poly(amino acid)s such as poly(L-aspartic acid) and poly(L-glutamic acid); or (iv) lipids like phosphoethanolamine (PE) (86, 87). Particular interest has been given to PEG-PPO block copolymers (poloxamers and poloxamines) (88, 89).They are commercialy accessible in a wide range of compositions and MW (Pluronic®/Lutrol®/Kolliphor® and Tetronic®).

2.2.1.3. Characteristics of amphiphilic copolymers and copolymer-based structures

a) Self-assembly

Amphiphilic polymers are able to the form nanoscopic structures of different morphologies, e.g. polymersomes, nanocapsules, nanospheres, nanogels or dendrimers, although polymeric micelles (PM) are the most commonly used and researched (85, 90, 91). Micellization into spherical nanosized particles with a hydrophilic corona and hydrophobic core occurs at or above a specific threshold level of polymer concentration (critical micellar concentration - CMC) and temperature (critical micellar temperature - CMT) (86, 87). In water this process is driven by an increase in entropy of the solvent molecules in contact to the hydrophobic units of the polymer and a consequent decrease of free energy (Δ°Gm) as the hydrophobic components are withdrawn from the aqueous media originating the micelle core (92). Δ°Gm is given by **Equation 2.1** where, R is the gas constant and T is the temperature of the system.

$$\Delta°\mathbf{Gm} = \mathbf{RT}\ln\mathbf{CMC} \qquad \textbf{Equation 2.1}$$

Different parameters such as chemical composition, MW, molecular architecture (linear versus branched, double bounds, etc.), concentration, temperature, solvent-polymer interactions, or salt concentration influence the self-assembly of polymers. By increasing the temperature of the system, the solvency of hydrophilic unit as well as the CMC value will decrease, promoting the micellization. Similarly, this phenomena is favored when the attractive hydrophobic interactions increases as a result of a gain in the MW of the hydrophobic domain (93). For example, PEG-b-PHOHH copolymers with PHOHH segments of 1500 and 7700 g/mol present a CMC value of 5.50 and 0.93 mg/L, respectively

(94). PM have been developed to modify several major intrinsic characteristics of drugs, including drug aqueous solubility, release pattern, pharmacokinetics, biodistribution, and *in vivo* stability (87). In general, the solubilisation capacity of hydrophobic drugs into micelles is proportional to the hydrophobicity of the micelle core (95). PM have an higher core hydrophobicity then surfactant micelles, conferring them higher thermodynamic and kinetical stability, thus presenting slower and delayed disintegration and drug release in circulation even upon dilution below the CMC value (84).

Table 2. 1. Examples of self-assembled particles under clinical trials evaluation.

Formulation	Copolymer	Drug	Indication	Clinical phase	Reference
SP1049C	Pluronic® L61 and F127	Doxorubicin	Advanced adenocarcinoma of the esophagus, gastroesophageal junction and stomach	III	(96)
Genexol-PM®	PEG-PLA	PTX	Breast cancer, non-small cell lung cancer, advanced pancreatic cancer and ovarian cancer	II/III/IV	(97-101)
NK012	PEG-poly(L-glutamic acid)-SN-38 conjugated	SN-38	Advanced breast cancer and small cell lung cancer	II	(102, 103)
NK105	PEG-poly(L-aspartic acid)	PTX	Advanced or recurrent breast cancer	III	(104)
NK-911	PEG–poly(aspartic acid)-doxorubicin conjugated	Doxorubicin	Solid tumors	I	(105)
NC-4016	PEG-DACH-platin	Oxaliplatin	Advanced solid tumors or lymphoma	I	(106)
NC-6004 (Nanoplatin®)	PEG-poly(L-glutamic acid)-cisplatin conjugated	Cisplatin	Solid tumors, non-small cell lung, biliary and bladder cancer, pancreatic cancer	II/III	(107, 108)
Paxceed®	PEG-PLA	PTX	Rheumatoid Arthritis, Psoriasis	II	(109, 110)
IT-101	Polymer-cyclodextrin-camptothecin conjugated	Camptothecin	Advanced solid tumors, ovarian cancer	I/II	(111, 112)
AquADEK®	D-alpha-tocopheryl-co-PEG 1000 succinate	Vitamins and antioxidants	Multivitamin supplement in Cystic fibrosis	II	(113)

DACH-platin: diaminocyclohexane platinum; PEG: polyethylene glycol; PLA: poly(d,l-lactide); PTX: paclitaxel

Due to their therapeutic efficacy, some PM-based formulations passed from the pre-clinical assessment to enroll clinical trials (**Table 2.1**) (114-117). One of them (Genexol-PM®, Samyang Co.) firstly approved in 2007 in Korea and being evaluated in European Union and America with the name Cynviloq® for the treatment of breast, non-small cell lung and ovarian cancers (118-120), while SP1049C (Surpatek Pharma, Inc.) granted orphan drug designation by FDA for the treatment of gastric cancer (121).

b) Surface hydrophilicity and functionalization

While the hydrophobic core of micelles, in addition to the solubilisation and protection of hydrophobic drugs, provides appropriate mechanical properties for the desired application; hydrophilic shell allows the encapsulation of hydrophilic compounds, and masks the particle from the biological environment, thereby reducing the protein absorption and cellular adhesion, and enhancing the particle stability (122, 123). Opsonin proteins present in the blood serum promptly bind to particles, allowing their recognition by macrophages and consequent elimination of the encapsulated drugs from bloodstream (124). The presence of a hydrophilic layer on the surface of particles will provide them with stealth properties by reducing or delaying the opsonization via steric repulsion forces, thus increasing the plasma circulation time of particles and the half-life of drugs (124, 125). PEG and poloxamers are commonly used as hydrophilic polymer to covers the surface of many nanoDDS creating the so called stealth systems (126). Moreover the resultant functionalization potential by either chemical modification or bioconjugation could be used to bind targeting moieties to the particles surface allowing a better control of the biodistribution of DDS (127). A variety of ligands such as peptides, antibodies, folate, transferrin or polysaccharides, can be used, accordingly to the required application.

c) Stimuli-responsive properties

Among all the characteristics presented by some amphiphilic polymers for the development of advanced controlled DDS, stimuli-responsiveness appears as one of the most interesting (128, 129). Stimuli-responsive polymers are a class of “smart” polymers that undergo chemical or physical alterations as response to a specific stimulus, e.g. temperature, pH, redox potential, magnetic field or light (129-131). Acrylate/methacrylate derivatives and monomers of N-alkyl substituted acrylamides are commonly used in the production of pH-responsive and thermo-responsive copolymers, respectively (129, 132). At acidic pH the existence of intermolecular polymer complexes lead to the formation of compact gels that

swells and became loose just at basic pH (133, 134). Multi-stimuli-responsive polymers (to light, temperature, and pH) with possible applications in biomedical field can be also developed (135).

2.2.1.4. Amphiphilic polymers-based gene delivery systems

Amphiphilic polymers have been proposed as gene carriers in the last years. As an example Guo S, *et al.* (2011) engineered ternary complexes by coating the surface of polycaprolactone-graft-poly(N,N-dimethylaminoethyl methacrylate) (PCL-g- PDMAEMA) nanoparticles/DNA with polyglutamic acid-graft-poly(ethylene glycol)(PGA-g-mPEG), in order to decrease the zeta potential of the system without interfere with DNA condensation ability. In *in vitro* studies, this non-covalent post-PEGylated ternary complexes presented high stability, low cytotoxicity, pronunced transfection efficiency, and improved DNA endosomal escape ability over the binary complexes. Moreover, *in vivo* results, namely histochemical analysis of tumor sections showed that the tumor expression of a red fluorescent protein encoded by the RFP plasmid is higher when the ternary complex was intravenously administrated (52).

In another study, Liu Y, *et al.* (2011) constructed a library of mono-methoxyl-poly(ethylene glycol)-block-poly(ε-caprolactone) (mPEG-PCL)-modified hyperbranched PEI copolymers (hy-PEI-PCL-mPEG) varying mPEG and PCL segments length, graft density, and hy-PEI MW in order to explore the influence of polymer compositions on siRNA delivery. Longer mPEG and PCL segments with higher graft density improve the stability and formation of polyplexes, reducing their cytotoxicity and zeta-potential. Hy-PEI-PCL-mPEG with very short PCL segments presented greater transfection efficiencies compared with those without PCL (hy-PEI25k) (136).

Amino acid-based polymers have also been proposed. In this context, PEO-PLAA-based structures emerge as an opportunity for the development of more effective therapeutic PM. The presence of carboxyl or amine groups in the PLAA block is advantageous for chemical conjugation and electrostatic interactions with drugs and nucleic acids and facilitates the chemical modification of the micellar core (85).

Poly(d,l-lactide) (PLA) and PLGA have also been formulated into micelles or nanoparticles and extensively used for sustained nucleic (137). Yang XZ, *et al.* (2011) produced a polymeric nanoparticle system for efficient siRNA encapsulation and delivery based on poly(ethylene glycol)-b-poly(d,l-lactide) (mPEG-PLA) and an amphiphilic cationic lipid (BHEM-cholesterol) by a non-condensation process and a double-emulsion solvent evaporation technique. *In vitro* assays showed that these nanoparticles allow efficient

siRNA cell internalization with further adequate endosomal escape, which results in the selective knockdown of the specific gene of interest, in this case, a significant downregulation of luciferase expression in HepG2-luciferase cells and suppression of polo-like kinase 1 (Plk1) expression in HepG2 cells. In addition, the nanoparticles encapsulating siRNA targeting the Plk1 gene showed to induce notable apoptosis in HepG2 and MDA-MB-435 cancer cell lines. *In vivo* studies on an orthotopic murine metastatic liver cancer model (MDA-MB-435 xenographs) further confirm the *in vitro* data, where luciferase expression was significantly inhibited and tumor growth suppressed after nanoparticles systemic administration (137). All these results suggest that mPEG-PLA nanoparticles have great potential for gene therapy. More recently, Sun J, *et al.* (2016) tested the amphiphilic triblock copolymer poly(hydroxyletheyl methacrylate-L-lysine)-b-poly(L-lactide)-b-poly(hydroxyletheyl methacrylate-L-lysine)s (PHML-b-PLA-b-PHML) to efficiently condensate a plasmid DNA and protect it from degradation by DNase I, and promoted a strong transfection efficiency without significant toxicity. The endocytosis of this particles seems to be through the lipid-raft-mediated endocytosis pathway, similar to that of SV40 virus-based vectors (138).

Murata N, *et al.* (2008) produced a biodegradable long-term sustained release PLGA microsphere through the water-in-oil-in-water drying method for encapsulation of anti-VEGF siRNA in order to suppress angiogenesis and tumor growth *in vivo*. Arginine or branched PEI was used as transfection agent/carrier for siRNA delivery. Using mice bearing S-180 tumors they demonstrated that after an intra-tumor injection of these microspheres VEGF gene expression and consequently the tumor growth were highly suppressed which indicate that this formulation is an useful approach to cancer treatment (139).

Worth to note is the research of Alshamsan A, *et al.* (2010), that investigated the incorporation of siRNA/PEI and siRNA/PEI-stA (the stearic derivative of PEI) complexes into PLGA nanoparticles for STAT3 knockdown in dendritic cells (DC). The *in vivo* and *in vitro* cytotoxicity that already had been detected for PEI or PEI-StA based polyplexes, was significantly reduced with this systems. They observed that the successful and specific STAT3 silencing is associated with the reestablishment of DC maturation and functionality, therefore this formulation represents an interesting strategy for targeted siRNA delivery to DCs and a promise for combined cancer immunotherapy (140).

2.2.1.5. Pluronic® and its role as biological response modifier

Pluronic® amphiphilic block copolymers have been shown high stability and the capacity to improve significantly the transfection efficiency and gene expression via different delivery

routes (24, 89, 141). The mechanism by which poloxamers enhance gene expression is not clearly understood but seems to be related to its ability to bind to cellular membranes and further activation of cell signalling pathways, such as the Nf-κB pathway. Nf-κB is a transcription factor able to bind pDNA in the cytoplasm and transport it to the nucleus using the nuclear transport machinery. Yang Z, *et al*. (2008) demonstrate for the first time that Pluronic® enhances both cellular uptake and nuclear transport of polyplex-delivered pDNA into NIH 3T3 cells, underling the role of poloxamers as a biological response modifier. On the other hand, poloxamer did not affect the naked DNA uptake, emphasizing the importance of delivering pDNA using polyplexes (24).

Another study from Kuo JH, *et al.* (2003) compared the serum stability of PEI/DNA and Pluronic-PEI/DNA complexes with different hydrophilic lipophilic balances (HLB) in terms of DNA delivery capacity to NIH/3T3 cells. The presence of Pluronic® especialy with higher HLB values, such as F68, conferred higher stability and increased significantly the gene expression (142). Also, Wang M, *et al.* (2014) investigated a series of small-size PEI-conjugated Pluronic® polycarbamates (PCMs) for the ability to modulate the delivery of 2′-O-methyl phosphorothioate RNA (2′-OMePS) both *in vitro* and in dystrophic (mdx) mice for the treatment of muscular dystrophy or other appropriate myodegenerative diseases. They conclude that the effective PCMs, especially those composed of moderate MW (2k–5kDa) and intermediate HLB of Pluronic®, enhanced exon-skipping of 2′-OMePS with low toxicity as compared with Lipofectamine® 2000 *in vitro* or PEI 25k *in vivo* (143).

Taking into account the different reports, the use of Pluronic® in the development of non-viral gene delivery systems seems to be a simple and safer approach that enhances both the *in vitro* and *in vivo* gene expression without inducing significant cytotoxic effects or inflammatory responses.

2.2.1.6. Polymer-based non-viral vectors on the way to clinical trials

The emerging advances observed in the area of gene delivery using polymeric nanoparticles technology, including the ones based in amphiphilic polymers, allowed the opportunity to bring these concepts to the clinical practice. In 2008, CALAA-01 becomes the first targeted delivery of siRNA to be tested in a phase I clinical trial to treat solid tumors refractory to standard therapies. CALAA-01 consists in a cyclodextrin-containing polymer (CDP)-based delivery system loaded with siRNA against ribonucleotide reductase subunit 2 (RRM2) and was design to inhibit tumor growth. The formulation also contains PEG as a stabilizing agent, and human transferrin (Tf) as a targeting ligand for binding to overexpressed transferrin receptors (TfR) of cancer cells (144). Besides CALAA-01,

Calando Pharmaceuticals already announced the development of CALAA-02, based on the same platform but encapsulating a siRNA against the HIF-2alpha.

Table 2. 2. Examples of polymeric nanoparticles for gene delivery at different stages of development.

Polymer vector	Genetic material	Utility	State	Reference
Dendritic PLL-block-poly(L-lactide)-block-dendritic PLL	pGFP	GFP expression	*in vitro*	(145)
CS	siRNA luc Gl3	Luciferase (luc) reporter gene downregulation	*in vitro*	(146)
Derivatives of low molecular- weight PEI	pGL3	Luciferase expression	*in vitro*	(147)
PEI-PEG-TAT peptide	pGFP-N3	GFP expression	*in vitro*	(148)
CTAB	p-ialB and p-omp25 DNA vaccines	Protection against *Brucella melitensis*	*in vivo*	(149)
CS oligomers	pCpG-Luc, gWiz-Luc, pCpG-GFP and gWiz-GFP	Luciferase and GFP expression	*in vivo*	(150)
ARC-520 (Dynamic PolyConjugates)	siRNA against apolipoprotein B and peroxisome proliferator-activated receptor alpha	Hepatitis B	*in vivo*	(151, 152)
PEG-PLL	sFlt-1 pDNA	Solid tumors	*in vivo*	(153)
PEG-poly[N'-[N-(2-aminoethyl)-2-aminoethyl]aspartamide]	pGL4.13 and sFlt-1 pDNA	Solid tumors	*in vivo*	(154, 155)
Pluronic® P85	pGFP	GFP expression	*in vivo*	(156)
CALAA-01	siRNA against ribonucleotide reductase M2	Melanoma	Phase I	(157, 158)
Polyvinylpyrrolidone	pIFN-alpha, pIL-12 and phIGF-1	Cubital tunnel syndrome, oropharyngeal cancer, squamous cell carcinoma and malignant melanoma	Phase I/II	(159-161)
PHML-b-PLLA-b-PHML	pDNA	Not tested for a specific disease	*in vitro*	(138)

CTAB: cetyltrimethylammonium bromide;PHML-b-PLLA-b-PHML: poly(hydroxyletheyl methacrylate-L-lysine)-b-poly(L-lactide)-b-poly(hydroxyletheyl methacrylate-L-lysine).

In the next years it is expected that new polymeric non-viral vectors based on amphiphilic polymers for both RNAi and DNA therapeutic strategies reach clinical evaluation, thus contributing for the progress of gene therapies in a variety of diseases. In **Table 2.2** are

summarized some of the many works proposed involving polymeric nanoparticles for gene delivery at different stages of development.

2.2.2. Lipid-based Delivery Systems (Lipoplexes)

Lipid-based non-viral vectors including liposomes, lipidic micelles, solid lipid nanoparticles (SLN), among others, became under the spotlight of many research teams as a strategy to overcome positively charge polyplexes toxicity and some possible lack of biocompatibility and/or biodegradability, while protecting the nucleic acid from nuclease degradation, allowing systemic delivery, and enhancing cellular uptake (162, 163).

Lipoplexes are usually produced through a simple interaction between nucleic acids and different lipids through different techniques such as the hydration of a lipid film, the dehydration-rehydration method, the ethanolic injection, the reverse-phase evaporation or the detergent dialysis technique (70).

Three types of lipids are usually selected to form the lipoplexes: i) anionic (e.g. cholesteryl hemisuccinate (CHEMS)), ii) neutral (e.g. 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphoethanolamine (DOPE) and cholesterol), and iii) cationic (e.g. 1,2-di-O-octadecenyl-3-trimethylammonium propane (DOTMA) or Lipofectin®, 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), 2,3-dioleyloxy-N-[2(sperminecarboxamido) ethyl]-N,N-dimethyl-1-propanaminium trifluoroacetate (DOSPA) or Lipofectamine®, 1,3-dioleoyloxy-2-(6-carboxyspermyl)-propyl amide (DOSPER), and Oligofectamine®).

The cationic lipids, due to its high capacity to complex the anionic ONG are commonly chosen. The final charge of the lipoplex, probably one of the most important factors affecting lipoplexes stability and transfection efficiency, depends on the ratio between the differently charged lipid vector and the nucleic acid molecules. Besides its charge and composition, the size and morphology of the lipoplexes also tremendously affect their transfection activity. Cationic lipoplexes, for example, does have a vast heterogeneity in terms of shape and size and different morphologic forms (such as cubic, beads on a string, spaghettis, meat balls, multilamellar, map-pin, sliding columnar and inverted hexagonal phase structures) have been reported (164). Regarding lipoplexes size, there is still a large controversy about which size range promotes better transfection activities. Some authors such as Esposito C, *et al*. (165) or Massoti A, *et al*. (166) advocate that lipoplexes within 200-400 nm or even 1-2 μm are more efficient, arguing that larger particles have a faster sedimentation process, present a higher contact with cells and form larger intracellular vesicles, that are more easily disrupted, improving the endosomal escape (165, 166). However, other authors report that higher transfection efficiencies are obtained using

particles with a smaller size of around 100 nm or less (167, 168). Although the optimal size for particles *in vitro* is still unclear, for the *in vivo* applications is of general consensus that small diameter nanoparticles are the more effective ones due to their avoidance from reticuloendothelial system and improved biodistribution (164).

The lipoplexes internalization by cells depends on the type of lipid used and the type of genetic material that is delivered, being reported two main mechanisms. The majority of the cases it is processed by endocytosis and in a low percentage of the cases by direct fusion with the cellular membrane. As example, Rejman J, *et al*. (169) demonstrated that DOTAP/DNA lipoplexes uptake occurs via clathrin-mediated endocytosis, while according to Wong AW, *et al*. (170), the uptake of a plasmid DNA associated with DMRIE-C transfection reagent occurs via the caveolin pathway. For synthetic siRNAs another mechanism was recently reported by Lu JJ, *et al*. (171), which showed that besides around 95% of siRNA lipoplexes enter the cells by endocytosis, the functional siRNA delivery occurs via minor pathways that involve simply the fusion with the plasma membrane. It was observed that when clathrin, caveolin or macropinocytosis mediated uptake is inhibited, the knockdown of the protein of interest continues to happen. On the other hand, when the cholesterol from the plasma membrane is depleted there are a significant reduction of the knockdown of the protein (171).

2.2.2.1. Cationic lipoplexes

Cationic lipids are generally amphiphilic molecules formed by a positively charged polar headgroup attached via a linker to a hydrophobic domain, which hydrocarbon chains lengths vary commonly between C8:0 and C18:1. The characteristics of the headgroup are responsible for the transfection efficiency of the lipoplexes; therefore several synthetic cationic lipids with variations in the headgroup composition have been produced. Headgroups consisting on amines groups with different substitution degrees, guanidinium or pyridinium groups, amino acids and peptides have been reported (9, 12, 13).

Despite their unquestionable advantages in terms of transfection efficiency, the toxicity of cationic lipids is a well-known problem that needs to be addressed. Eventhough, Lipofectamine® 2000 is widely used as transfection reagent in mechanistic studies and gold standard to assess the efficacy of other systems. Therefore, with the final objective of reaching an ideal gene delivery system with minimal toxicity and adequate transfection efficiency, numerous cationic lipids based formulations have been continuously designed and produced, presenting adequate *in vitro* and *in vivo* results and some of them enrolled already clinical assessment (**Table 2.3**). These new cationic lipids are obtained through

structural modifications performed in the headgroup, in the linker and/or in the hydrophobic tail domains that are known to regulate transfection efficiency and cytotoxicity (172).

2.2.2.2. Anionic and neutral lipids

The use of anionic or neutral lipids as gene delivery systems is another strategy that some authors explore with the objective of override the cationic lipids associated toxicity. However, due to their negative or neutral charge and the consequent impossibility to establish electrostatic interactions with negatively charged molecules, the delivery systems based on anionic or neutral lipids always require the incorporation of a third moiety such as divalent or monovalent cations (Ca^{2+}, Na^{+}, Mg^{2+}, Mn^{2+} or Ba^{2+}) to promote the complexation between the nucleic acids and the lipids (173-175).

Kapoor M, *et al*. (175) designed a siRNA delivery system using physiologically occurring anionic lipids (DOPG/DOPE) and calcium ions bridges. The obtained lipoplexes presented high stability in the presence of serum components, efficient cellular uptake and effective endosomal release, and a significant silencing capacity comparable with the control cationic complexes (Lipofectamine® 2000), showing lower toxicity (175).

In another study, Mignet N, *et al*. (173) proposed an innovative system throughout the combination of a cationic lipoplex and pegylated anionic liposomes. The ratio between anionic and cationic lipids was optimized in order to form an anionic pegylated lipoplex sensitive to pH changes (5.5-6.5). Reversion to a cationic net charge at an adequate pH value was observed during endosomal acidification which leads to the DNA release and consequent increase of the gene expression. These result was comparable with the control CHEMS formulation and more importantly, showed an improved ability to deliver DNA to tumor tissues (173).

2.2.3. Lipid Nanoparticles

Different types of liposomes (cationic, neutral and anionic) have been developed and successfully used for nucleic acids administration (57, 176, 177). Among them, Stable Nucleic Acid Lipid Particles (SNALPs) have been enjoying special interest for the treatment of several diseases. They consist in a lipid bilayer system composed by a mixture of cationic and fusogenic lipids shielded with PEG to provide a neutral hydrophilic surface, avoiding opsonization and rapid systemic clearance *in vivo*, as well as allowing an efficient particle targeting **(Figure 2.7)** (162, 178-182). Neutral lipids such as DOPE, cholesterol or (1,2-

dioleoyl-sn-glycero-3-phosphocholine) (DOPC), also called helper lipids, are many times added to the system, since play an important role improving particles robustness and physiologic stability (9, 183, 184).

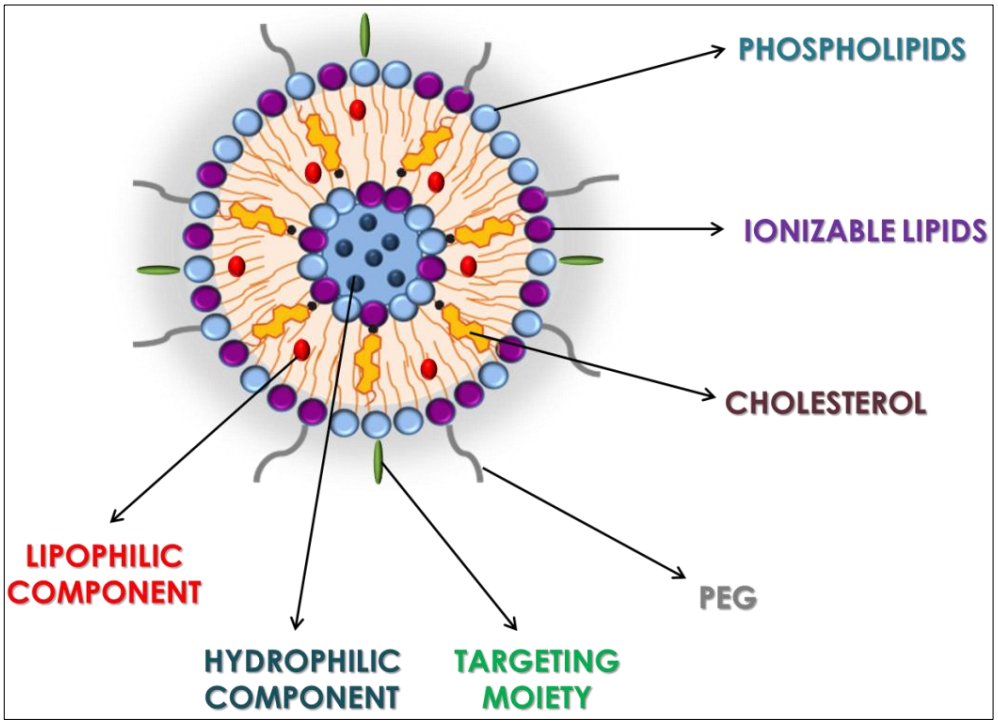


Figure 2. 7. Schematic representation of a SNALP and its main components. PEG: polyethylene glycol

For example, TKM-PLK1 and ALN-VPS are both SNALP-based siRNA systems that recently enrolled in clinical trials (185, 186).

Despite the favorable biocompatibility profiles of the majority of the components presented in liposomes, some major concerns arise mainly related to the cationic reactivity-related toxicity and lower entrapment efficiency presented by many of the proposed systems (162). Regarding the usual drawbacks related with cationic liposomes such as their cytotoxicity, non-specific interactions with negatively charged cellular components, short half-time life and high immunogenicity, many efforts have been done to circumvent these problems. As an example, Semple SC, *et al.* (2001) developed liposomes using the pH-sensitive ionizable aminolipid DOTAP, presenting a neutral charge at physiological pH, that becomes cationic at the endosomal pH (187).

Another common strategy is based in the addition of neutral lipids such as DOPC, DOPE, 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and phosphocholine (PC) to reduce the total cationic surface charge (163, 188). Atu027 (based on AtuPLEX® system), is an

example of this approach, a cationic liposomal formulation containing neutral fusogenic lipids, designed for delivery of siRNA against the protein kinase PKN3. In phase I clinical trials, the system has proved to be well tolerated in the administered doses, being a hopefull antiangiogenesis therapeutic strategy for the treatment of advanced solid tumors (189).

Apart from liposomes, SLN have also been proposed as non-viral vectors due to their proposed advantages such as high stability, protection of the encapsulated agents, high entrapment ability, small and uniform sizes, easily modulated via the composition, sustained controlled release properties, biodegradability, ease manufacture and scale up at relatively low cost, or suitability for sterilization and lyophilization. They showed to be capable of encapsulating OGN, mainly using plasmids as a model, in the lipid core or on the particle surface (190).

Bondi L, *et al*. (2007), proved the suitability of cationic SLN based on acylglicerol lipids, able to form stable complexes with DNA and to protect DNA against DNase I digestion. The *in vitro* studies performed on human liver cancer cells also demonstrated a low degree of toxicity of both SLN and SLN–DNA complexes (191). With the objective to assess the *in vivo* transfection potential of SLN, Rodríguez A, *et al*. (2010), demonstrated for the first time the capacity of SLN-DNA vectors to induce the expression of a foreign protein in mice. They use SLN as a vector to transfect the pCMS-GFP plasmid that encodes the GFP and observed protein expression in hepatic and splenic tissues (192).

Regarding siRNA delivery, Lobovkina T, *et al*. (2011), demonstrated that SLN can incorporate and provide a sustained release of siRNA. SLN were intradermic injected in mouse footpads and a prolonged siRNA release over a period of 10-13 days was observed, with retention of the siRNA activity (193). In another study, Yu Y, *et al*. (2012), proposed a formulation of cationic SLN for co-delivery of PTX and human myeloid leukemia cell differentiation protein (MCL1)-specific siRNA (siMCL1). This system significantly reduced the MCL1 mRNA levels in KB cells, contrary to the group treated with siMCL1 alone. Additionally, the *in vivo* results shown significant reduction of the tumor growth in KB cell-xenografted mice (194).

Some examples of the lipidic non-viral vectors for gene delivery are summarized in **Table 2.3.**

Table 2. 3. Examples of lipidic nanoparticles for gene delivery at different stages of development.

Lipid vector	Genetic material	Utility	State	Reference
LinOS/cholesterol	GFP siRNA	GFP delivery and silencing	*in vitro*	(195)
PEGylated liposomes	Surviving siRNA	Tumor reduction	*in vivo*	(167)
PEGylated lipossomes	Argonaute2 siRNA	Anti-angiogenesis effect and tumor growth suppression	*in vivo*	(196)
DOTMA	CpG DNA	Prevent pulmonary metastasis and peritoneal dissemination through via NK cell activation.	*in vivo*	(197)
PLAS	Lamin A/C siRNA	Sustained delivery of siRNA to vaginal epithelium.	*in vivo*	(198)
PEI-PEGylated liposomes	AML1/MTG8	Decrease leukemic clonogenicity in CD33-positive acute myeloid leukemia cells	*in vivo*	(199)
Atu027 (AtuPLEX® system)	Protein kinase N3 siRNA	Advanced solid tumor and pancreatic cancer	Phase I/II	(200, 201)
DMRIE/DOPE	pGT-1 gene	Cystic fibrosis	Phase I	(202)
DOTAP/cholesterol	Fus 1 gene	Non-small-cell lung cancer	Phase I/II	(203, 204)
SNALP (ALN-PCL)	PCSK9	Hypercholesterolemia	Phase I	(186)
SNALP (TKM-PLK1)	PLK1	Refractory neuroendocrine tumors, adrenocortical carcinoma and metastatic cancers	Phase I/II	(205, 206)
QPI-1002	p53 siRNA	Acute kidney injury and prevention of delayed graft function	Phase II/III	(207, 208)
PF-655	RTP801 siRNA	Diabetic macular edema and wet age-related macular degeneration	Phase II	(209)

DMRIE: 2,3-di(tetradecoxy)propyl-(2-hydroxyethyl)-dimethylazanium bromide; DOPE: 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphoethanolamine; DOTAP: 1,2-dioleoyl-3-trimethylammonium-propane; DOTMA: 1,2-di-O-octadecenyl-3-trimethylammonium propane; LinOS: N4-linoleoyl-N9-oleoyl-1,12-diamino-4,9-diazadodecane; NK: natural killer; PCSK9: Proprotein convertase subtilisin/kexin type 9; PLAS: PEGylated lipoplex-entrapped alginate scaffold; PLK1: polo-like kinase 1.

2.2.4. Combination of Polymers and Lipids: does it meet the ideal system?

In order to benefit from the advantages of both polymeric and lipidic systems, Lipopolyplexes (LPP) **(Figure 2.8)** i.e. ternary complexes composed essentially by cationic lipids, a cationic polymer/peptide, and nucleic acids, were conceived (210-213). This hybrid

system combine the delivery capabilities of cationic liposomes with the advantages of cationic polymers/peptides given rise to a more efficient complexation, delivery and transfection efficiencies compared to the first generation ones represented by lipoplexes and polyplexes (211, 212, 214-222). Several authors have reported an additional OGN protection against enzymatic degradation, and superior physical stability (223), reduced cytotoxicity (224, 225) as well as an elevated transfection efficiency (211) when the nucleic acid is incorporated in a polymer-lipid complex.

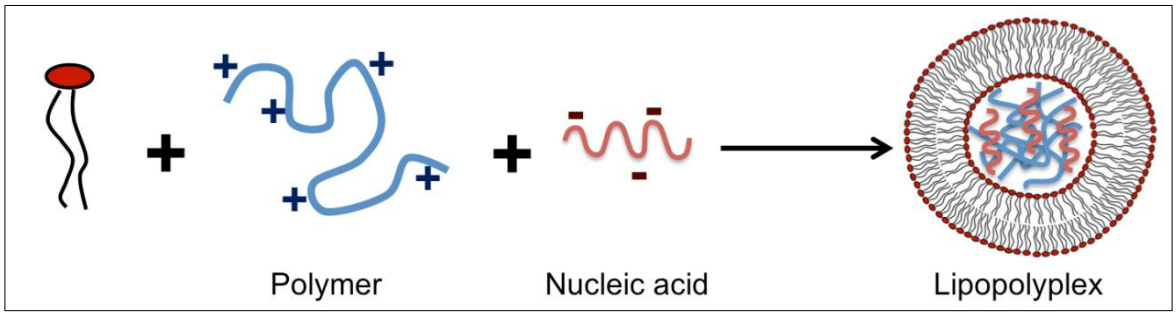


Figure 2. 8. Schematic representation of a type of lipopolyplex and its main components.

PEI is one the cationic polymers highly proposed to be complexed with lipoplexes to form the LLP (214, 216, 226, 227). Kurosaki T, *et al*. (2009), have developed a LPP formulation for pulmonary gene delivery composed of PEI, DOTMA and pDNA (227). Markedly transfection efficiencies in HepG2 cells, without any cytotoxicity or aggregation with erythrocytes were proved. Moreover, further *in vivo* studies revealed high levels of transfection efficiency in lung cells after intravenous administration (227). A similar LPP formulation combined with folic acid at the surface of the system significantly enhanced its transfection efficiency and gene-silencing activity without a significant cytotoxic effect (213). Pelisek J, *et al*. (2006), investigated a combination of linear and branched PEI with liposomes of DOSPER and DOTAP for gene transfer to slow-proliferating human colon carcinoma cell lines (214). In all cases, LPP were between 5 to 400 times more efficient compared to the corresponding lipoplexes or polyplexes, being therefore favorable nanocarriers for *in vitro* and *in vivo* gene transfer in colorectal cancer cells (214).

Another system taking profit of the LPP advantage was proposed by Alshamsan A, *et al*. (2011) for the knockdown of the signal transducer and activator of transcription 3 (STAT3) through the use of stearic acid modified with PEI. This system was tested in B16 melanoma *in vitro* and *in vivo*, showing good results namely in terms of a significant increase in IFN-γ, IL-2 and IL-12, and TNF-α within the tumor microenvironment (228).

Regarding LPP complexes compromising liposomes, nucleic acids and cationic peptides, the most commonly used peptide is protamine (215, 217, 219, 221, 223, 229), but lysine

(215, 230-232), arginine and histidine (222, 231) have also been studied. The strong association between peptides and nucleic acids confers to such LPP particles an enhanced transfection efficiency and gene silencing. Moreover, this complexation promotes the capacity to transfect cells in the presence of serum (184, 233). Cationic lipid-protamine-OGN complexes have presented an increased OGN protection against enzymatic digestion, a superior physical stability, and higher gene expression in cancer cells after intravenous administration (219, 223, 229). Li SD & Huang L (219) and Tseng YC, *et al*. (2009) have developed a tumor-targeted LPP for siRNA delivery (234). The nucleic acid was condensed with protamine and the resulting nanoparticles were coated with DOTAP and cholesterol (219, 234). Further PEGylation of the LPP complex significantly increased the tumor localization of siRNA by four-fold, while a two- to three-fold increase in the silencing effect was observed in an *in vivo* human lung cancer model (219, 220).

Anionic lipids have also been used for the development of LPP systems, comprising pH-sensitive liposome and protamine, for macrophage gene therapy. *In vitro* tests revealed a safety profile and increased transfection efficiency of LPP compared to Lipofectamine® 2000 or protamine/DNA complexes (235).

Another type of LPP nanocarriers is the pegylated immuno-lipopolyplexes (PILP). In these complexes, the nucleic acids are firstly compacted by a cationic polymer, and complexed with anionic liposomes to produce the LPP. The surface of LPP is covered with PEG, in order to promote stabilization in the bloodstream, and the PEG strands are functionalized with targeting moieties. The system (mAb-PEG2000/DSPE-PEI/DNA) developed by Hu Y, *et al*. (2010) demonstrated to be highly effective in protecting DNA from degradation, and to accumulate at the liver promoting a high efficiency in gene delivery and gene silencing in cancer cells without significant cytokines production or liver injury (236-238). LPP have also been described as an efficient system for mRNA (210, 218, 221, 222, 239) and DNA-based cancer vaccines (217, 218, 240).

Mockey M, *et al*. (2007) has developed a LPP for the delivery of mRNA encoding a tumor antigen (MART-1) against the progression of B16F10 melanoma. MART-1 was condensed in 50 nm particles of PEGylated histidylated PLL and then added to liposomes prepared with L-histidine-(N,N-di-n-hexadecylamine)ethylamide and cholesterol. Intravenous administration of this formulation induced the production of IFN-γ and the activation of cytotoxic T lymphocytes. No effect was observed when the mRNA was complexed with liposomes or with the cationic polymer alone (222). A similar nanosystem for the vaccination of B16F10 melanoma was also developed adding mannosylated and histidylated liposomes to mRNA-PEGylated histidylated PLL polyplexes. Authors were able to enhance the transfection of tumor antigen mRNA in splenic dendritic cells *in vivo* inducing therefore an

anticancer immune response (210, 239).

A DNA-based vaccine approach was proposed by Whitmore MM, *et al*. (241) consisting of DOTAP:cholesterol cationic liposomes, protamine sulfate, and pDNA with unmethylated CpG motifs. This complex was capable to stimulate a potent Th-1 cytokine response producing TNF-α, IL-12, and IFN-γ, and was also able to trigger the antitumor NK activity. The combination of these two innate immune responses was effective in inhibiting the growth of established tumors in mice as they provide an acquired tumor-specific cytotoxic T cell response (241).

LPP stability, efficacy and lack of toxicity justify their fostering arriving to clinical trials. In fact, EGEN-001 is a LPP formulation that undergoes phase I and II clinical trials, composed by IL-12 plasmid formulated in PEG-PEI-cholesterol to treat a variety of cancers such as ovarian, peritoneal, fallopian tube, and colorectal cancer (242-245).

2.3. Conclusions

Despite viral vectors are still the most common systems for gene therapy in clinical trials and the only ones that already reach the clinical practice, there are no doubts that the application of non-viral vectors for gene delivery was an amazing and essential discovery of the last years. Gene therapy mediated by lipoplexes, polyplexes or lipopolyplexes is a field that is clearly growing both in basic research and pre-clinical stages. It is expected that very soon new formulations enter in clinical trials while others grant market authorization for a variety of diseases. Given the chance to promote the silencing of different oncogenes or correct the expression of other absent or mutant genes, among the different healthcare areas to which new non-viral vectors based delivery systems are being developed, oncology is the one where research work is invested and where greater applicability is predictable.

Although lipopolyplexes seems to overcome some of the major drawbacks presented by the other systems, the assumption that an ideal non-viral system is far from being reached, lead us to recognize a pressing and urgent need for innovative and improved approaches. New advances should help to meet quality requirements, which demands a balance between effectiveness, safety and technological possibilities, aiming market approval. Attempting this balance with non-viral vectors is a worthy effort to meet critical patients needs.

2.4. References

1. Cavazzana-Calvo M, Hacein-Bey S, de Saint Basile G, Gross F, Yvon E, Nusbaum P, et al. Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. Science. 2000;288(5466):669-72.

2. Blaese RM, Culver KW, Miller AD, Carter CS, Fleisher T, Clerici M, et al. T lymphocyte-directed gene therapy for ADA- SCID: initial trial results after 4 years. Science. 1995;270(5235):475-80.

3. Kumar SR, Markusic DM, Biswas M, High KA, Herzog RW. Clinical development of gene therapy: results and lessons from recent successes. Mol Ther Methods Clin Dev. 2016;3:16034.

4. Kaufmann KB, Büning H, Galy A, Schambach A, Grez M. Gene therapy on the move. EMBO Mol Med. 2013;5(11):1642-61.

5. Wang D, Gao G. State-of-the-art human gene therapy: Part I. gene delivery technologies. Discov Med. 2014;18(97):67-77.

6. Videira M, Arranja A, Rafael D, Gaspar R. Preclinical development of siRNA therapeutics: towards the match between fundamental science and engineered systems. Nanomedicine. 2014;10(4):689-702.

7. Hacein-Bey-Abina S, von Kalle C, Schmidt M, Le Deist F, Wulffraat N, McIntyre E, et al. A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. N Engl J Med. 2003;348(3):255-6.

8. EMA. Glybera. EPAR - Product information. www.ema.europa.eu2012.

9. Wasungu L, Hoekstra D. Cationic lipids, lipoplexes and intracellular delivery of genes. J Control Release. 2006;116(2):255-64.

10. Eliyahu H, Joseph A, Schillemans JP, Azzam T, Domb AJ, Barenholz Y. Characterization and in vivo performance of dextran-spermine polyplexes and DOTAP/cholesterol lipoplexes administered locally and systemically. Biomaterials. 2007;28(14):2339-49.

11. Liu F, Huang L. Development of non-viral vectors for systemic gene delivery. J Control Release. 2002;78(1-3):259-66.

12. Zhang XX, McIntosh TJ, Grinstaff MW. Functional lipids and lipoplexes for improved gene delivery. Biochimie. 2012;94(1):42-58.

13. Zhang S, Xu Y, Wang B, Qiao W, Liu D, Li Z. Cationic compounds used in lipoplexes and polyplexes for gene delivery. J Control Release. 2004;100(2):165-80.

14. Scholz C, Wagner E. Therapeutic plasmid DNA versus siRNA delivery: common and different tasks for synthetic carriers. J Control Release. 2012;161(2):554-65.

15. Li L, Wei Y, Gong C. Polymeric Nanocarriers for Non-Viral Gene Delivery. J Biomed Nanotechnol. 2015;11(5):739-70.

16. de Martimprey H, Vauthier C, Malvy C, Couvreur P. Polymer nanocarriers for the delivery of small fragments of nucleic acids: oligonucleotides and siRNA. Eur J Pharm Biopharm. 2009;71(3):490-504.

17. Gary DJ, Puri N, Won YY. Polymer-based siRNA delivery: perspectives on the fundamental and phenomenological distinctions from polymer-based DNA delivery. J Control Release. 2007;121(1-2):64-73.

18. Mao S, Sun W, Kissel T. Chitosan-based formulations for delivery of DNA and siRNA. Adv Drug Deliv Rev. 2010;62(1):12-27.

19. Oskuee RK, Philipp A, Dehshahri A, Wagner E, Ramezani M. The impact of carboxyalkylation of branched polyethylenimine on effectiveness in small interfering RNA delivery. J Gene Med. 2010;12(9):729-38.

20. Chira S, Jackson CS, Oprea I, Ozturk F, Pepper MS, Diaconu I, et al. Progresses towards safe and efficient gene therapy vectors. Oncotarget. 2015;6(31):30675-703.

21. Xiong XB, Falamarzian A, Garg SM, Lavasanifar A. Engineering of amphiphilic block copolymers for polymeric micellar drug and gene delivery. J Control Release. 2011;155(2):248-61.

22. Ozpolat B, Sood AK, Lopez-Berestein G. Nanomedicine based approaches for the delivery of siRNA in cancer. Journal of internal medicine. 2010;267(1):44-53.

23. Dohmen C, Edinger D, Fröhlich T, Schreiner L, Lächelt U, Troiber C, et al. Nanosized Multifunctional Polyplexes for Receptor-Mediated SiRNA Delivery. ACS Nano. 2012.

24. Yang Z, Sahay G, Sriadibhatla S, Kabanov AV. Amphiphilic block copolymers enhance cellular uptake and nuclear entry of polyplex-delivered DNA. Bioconjug Chem. 2008;19(10):1987-94.

25. Dufresne MH, Elsabahy M, Leroux JC. Characterization of polyion complex micelles designed to address the challenges of oligonucleotide delivery. Pharm Res. 2008;25(9):2083-93.

26. Howard KA. Delivery of RNA interference therapeutics using polycation-based nanoparticles. Advanced drug delivery reviews. 2009;61(9):710-20.

27. Maeda H. The enhanced permeability and retention (EPR) effect in tumor vasculature: the key role of tumor-selective macromolecular drug targeting. Adv Enzyme Regul. 2001;41:189-207.

28. Misra R, Acharya S, Sahoo SK. Cancer nanotechnology: application of nanotechnology in cancer therapy. Drug Discovery Today. 2010;15(19-20):842-50.

29. Kim KY. Nanotechnology platforms and physiological challenges for cancer therapeutics. Nanomedicine : nanotechnology, biology, and medicine. 2007;3(2):103-10.
30. Vasir JK, Labhasetwar V. Biodegradable Nanoparticles for Cytosolic Delivery of Therapeutics. Advanced Drug Delivery Reviews. 2007;59(8):718-28.
31. Mathur V, Satrawala Y, Rajput MS, Kumar P, Shrivastava P, Vishvkarma A. Solid lipid nanoparticles in cancer therapy. International Journal of Drug Delivery. 2010;2(3):192-9.
32. Videira MA, Botelho MF, Santos AC, Gouveia LF, de Lima JJ, Almeida AJ. Lymphatic uptake of pulmonary delivered radiolabelled solid lipid nanoparticles. Journal of drug targeting. 2002;10(8):607-13.
33. Videira MA, Gano L, Santos C, Neves M, Almeida AJ. Lymphatic uptake of lipid nanoparticles following endotracheal administration. Journal of microencapsulation. 2006;23(8):855-62.
34. Pathak A, Patnaik S, Gupta KC. Recent trends in non-viral vector-mediated gene delivery. Biotechnology Journal. 2009;4(11):1559-72.
35. Zhang S, Zhao B, Jiang H, Wang B, Ma B. Cationic lipids and polymers mediated vectors for delivery of siRNA. Journal of Controlled Release. 2007;123(1):1-10.
36. Aigner A. Delivery Systems for the Direct Application of siRNAs to Induce RNA Interference (RNAi) In Vivo. Journal of Biomedicine and Biotechnology. 2006;2006:1-16.
37. Zimmermann TS, Lee ACH, Akinc A, Bramlage B, Bumcrot D, Fedoruk MN, et al. RNAi-mediated gene silencing in non-human primates. Nature. 2006;441(7089):111-4.
38. Martin I, Dohmen C, Mas-Moruno C, Troiber C, Kos P, Schaffert D, et al. Solid-phase-assisted synthesis of targeting peptide-PEG-oligo(ethane amino)amides for receptor-mediated gene delivery. Org Biomol Chem. 2012;10(16):3258-68.
39. Tahara K, Sakai T, Yamamoto H, Takeuchi H, Kawashima Y. Establishing chitosan coated PLGA nanosphere platform loaded with wide variety of nucleic acid by complexation with cationic compound for gene delivery. International Journal of Pharmaceutics. 2008;354(1-2):210-6.
40. Cho K, Wang X, Nie S, Chen Z, Shin DM. Therapeutic Nanoparticles for Drug Delivery in Cancer. Clinical Cancer Research. 2008;14(5):1310-6.
41. Tros de Ilarduya C, Sun Y, Düzgüneş N. Gene delivery by lipoplexes and polyplexes. European Journal of Pharmaceutical Sciences. 2010;40(3):159-70.
42. Wu GY, Wu CH. Receptor-mediated in vitro gene transformation by a soluble DNA carrier system. J Biol Chem. 1987;262(10):4429-32.
43. Dizaj SM, Jafari S, Khosroushahi AY. A sight on the current nanoparticle-based gene delivery vectors. Nanoscale Res Lett. 2014;9(1):252.

44. Nayerossadat N, Maedeh T, Ali PA. Viral and nonviral delivery systems for gene delivery. Adv Biomed Res. 2012;1:27.

45. Midoux P, Pichon C, Yaouanc JJ, Jaffrès PA. Chemical vectors for gene delivery: a current review on polymers, peptides and lipids containing histidine or imidazole as nucleic acids carriers. Br J Pharmacol. 2009;157(2):166-78.

46. Asseline U, Gonçalves C, Pichon C, Midoux P. Improved nuclear delivery of antisense 2'-Ome RNA by conjugation with the histidine-rich peptide H5WYG. J Gene Med. 2014;16(7-8):157-65.

47. Puras G, Martínez-Navarrete G, Mashal M, Zárate J, Agirre M, Ojeda E, et al. Protamine/DNA/Niosome Ternary Nonviral Vectors for Gene Delivery to the Retina: The Role of Protamine. Mol Pharm. 2015;12(10):3658-71.

48. Tao J, Ding WF, Che XH, Chen YC, Chen F, Chen XD, et al. Optimization of a cationic liposome-based gene delivery system for the application of miR-145 in anticancer therapeutics. Int J Mol Med. 2016;37(5):1345-54.

49. Johnson RP, Uthaman S, John JV, Lee HR, Park IK, Kim I. Biodegradable poly(ethylene glycol) methyl ether acrylate-b-poly(l-lysine)-b-poly(l-histidine) triblock copolypeptides for non-viral gene delivery. J Control Release. 2015;213:e93-4.

50. Feng T, Tian H, Dong X, Lam MH, Liang H, Chen X. pH-sensitive OEI-poly(aspartic acid-b-lysine) as charge shielding system for gene delivery. J Control Release. 2015;213:e104.

51. Raad M, Teunissen EA, Mastrobattista E. Peptide vectors for gene delivery: from single peptides to multifunctional peptide nanocarriers. Nanomedicine (Lond). 2014;9(14):2217-32.

52. Guo S, Huang Y, Zhang W, Wang W, Wei T, Lin D, et al. Ternary complexes of amphiphilic polycaprolactone-graft-poly (N,N-dimethylaminoethyl methacrylate), DNA and polyglutamic acid-graft-poly(ethylene glycol) for gene delivery. Biomaterials. 2011;32(18):4283-92.

53. Huang Y, Lin D, Jiang Q, Zhang W, Guo S, Xiao P, et al. Binary and ternary complexes based on polycaprolactone-graft-poly (N, N-dimethylaminoethyl methacrylate) for targeted siRNA delivery. Biomaterials. 2012;33(18):4653-64.

54. Guo S, Huang Y, Wei T, Zhang W, Wang W, Lin D, et al. Amphiphilic and biodegradable methoxy polyethylene glycol-block-(polycaprolactone-graft-poly(2-(dimethylamino)ethyl methacrylate)) as an effective gene carrier. Biomaterials. 2011;32(3):879-89.

55. Katas H, Alpar HO. Development and characterisation of chitosan nanoparticles for siRNA delivery. Journal of Controlled Release. 2006;115(2):216-25.

56. Nagpal K, Singh SK, Mishra DN. Chitosan nanoparticles: a promising system in novel drug delivery. Chem Pharm Bull (Tokyo). 2010;58(11):1423-30.

57. Wang X, Wang Y, Chen ZG, Shin DM. Advances of Cancer Therapy by Nanotechnology. Cancer Research and Treatment. 2009;41(1):1.

58. Liu X, Howard KA, Dong M, Andersen MO, Rahbek UL, Johnsen MG, et al. The influence of polymeric properties on chitosan/siRNA nanoparticle formulation and gene silencing. Biomaterials. 2007;28(6):1280-8.

59. Mao S, Sun W, Kissel T. Chitosan-based formulations for delivery of DNA and siRNA☆. Advanced Drug Delivery Reviews. 2010;62(1):12-27.

60. Kim TH, Kim SI, Akaike T, Cho CS. Synergistic effect of poly(ethylenimine) on the transfection efficiency of galactosylated chitosan/DNA complexes. J Control Release. 2005;105(3):354-66.

61. Wong K, Sun G, Zhang X, Dai H, Liu Y, He C, et al. PEI-g-chitosan, a novel gene delivery system with transfection efficiency comparable to polyethylenimine in vitro and after liver administration in vivo. Bioconjug Chem. 2006;17(1):152-8.

62. Kim TH, Ihm JE, Choi YJ, Nah JW, Cho CS. Efficient gene delivery by urocanic acid-modified chitosan. J Control Release. 2003;93(3):389-402.

63. Kiang T, Bright C, Cheung CY, Stayton PS, Hoffman AS, Leong KW. Formulation of chitosan-DNA nanoparticles with poly(propyl acrylic acid) enhances gene expression. J Biomater Sci Polym Ed. 2004;15(11):1405-21.

64. Moreira C, Oliveira H, Pires LR, Simões S, Barbosa MA, Pêgo AP. Improving chitosan-mediated gene transfer by the introduction of intracellular buffering moieties into the chitosan backbone. Acta Biomater. 2009;5(8):2995-3006.

65. Hu FQ, Zhao MD, Yuan H, You J, Du YZ, Zeng S. A novel chitosan oligosaccharide-stearic acid micelles for gene delivery: properties and in vitro transfection studies. Int J Pharm. 2006;315(1-2):158-66.

66. Shim MS, Kwon YJ. Efficient and targeted delivery of siRNA in vivo. FEBS Journal. 2010;277(23):4814-27.

67. Aigner A. Gene silencing through RNA interference (RNAi) in vivo: strategies based on the direct application of siRNAs. Journal of biotechnology. 2006;124(1):12-25.

68. Sorkin A, Von Zastrow M. Signal transduction and endocytosis: close encounters of many kinds. Nature reviews Molecular cell biology. 2002;3(8):600-14.

69. Akinc A, Thomas M, Klibanov AM, Langer R. Exploring polyethylenimine-mediated DNA transfection and the proton sponge hypothesis. J Gene Med. 2005;7(5):657-63.

70. Tros de Ilarduya C, Sun Y, Düzgüneş N. Gene delivery by lipoplexes and polyplexes. Eur J Pharm Sci. 2010;40(3):159-70.

71. Roesler S, Koch FP, Schmehl T, Weissmann N, Seeger W, Gessler T, et al. Amphiphilic, low molecular weight poly(ethylene imine) derivatives with enhanced stability for efficient pulmonary gene delivery. J Gene Med. 2011;13(2):123-33.

72. Godbey WT, Wu KK, Mikos AG. Size matters: molecular weight affects the efficiency of poly(ethylenimine) as a gene delivery vehicle. J Biomed Mater Res. 1999;45(3):268-75.

73. Thomas M, Klibanov AM. Enhancing polyethylenimine's delivery of plasmid DNA into mammalian cells. Proc Natl Acad Sci U S A. 2002;99(23):14640-5.

74. Mao S, Neu M, Germershaus O, Merkel O, Sitterberg J, Bakowsky U, et al. Influence of polyethylene glycol chain length on the physicochemical and biological properties of poly(ethylene imine)-graft-poly(ethylene glycol) block copolymer/SiRNA polyplexes. Bioconjug Chem. 2006;17(5):1209-18.

75. Dehshahri A, Oskuee RK, Shier WT, Hatefi A, Ramezani M. Gene transfer efficiency of high primary amine content, hydrophobic, alkyl-oligoamine derivatives of polyethylenimine. Biomaterials. 2009;30(25):4187-94.

76. Grayson AC, Doody AM, Putnam D. Biophysical and structural characterization of polyethylenimine-mediated siRNA delivery in vitro. Pharm Res. 2006;23(8):1868-76.

77. Meiners S, Brinkmann V, Naundorf H, Birchmeier W. Role of morphogenetic factors in metastasis of mammary carcinoma cells. Oncogene. 1998;16:9-20.

78. Merdan T, Callahan J, Petersen H, Kunath K, Bakowsky U, Kopeckova P, et al. Pegylated polyethylenimine-Fab' antibody fragment conjugates for targeted gene delivery to human ovarian carcinoma cells. Bioconjugate chemistry. 2003;14(5):989-96.

79. Malek A, Czubayko F, Aigner A. PEG grafting of polyethylenimine (PEI) exerts different effects on DNA transfection and siRNA-induced gene targeting efficacy. Journal of Drug Targeting. 2008;16(2):124-39.

80. Philipp A, Zhao X, Tarcha P, Wagner E, Zintchenko A. Hydrophobically modified oligoethylenimines as highly efficient transfection agents for siRNA delivery. Bioconjugate Chemistry. 2009;20(11):2055-61.

81. Guo G, Zhou L, Chen Z, Chi W, Yang X, Wang W, et al. Alkane-modified low-molecular-weight polyethylenimine with enhanced gene silencing for siRNA delivery. Int J Pharm. 2013;450(1-2):44-52.

82. Dong X, Lin L, Chen J, Guo Z, Tian H, Li Y, et al. A Serum-Tolerant Hydroxyl-Modified Polyethylenimine as Versatile Carriers of pDNA/siRNA. Macromol Biosci. 2013;13(4):512-22.

83. Kataoka K, Harada A, Nagasaki Y. Block copolymer micelles for drug delivery: design, characterization and biological significance. Advanced Drug Delivery Reviews. 2001;47(1):113-31.

84. Adams ML, Lavasanifar A, Kwon GS. Amphiphilic block copolymers for drug delivery. J Pharm Sci. 2003;92(7):1343-55.

85. Xiong XB, Binkhathlan Z, Molavi O, Lavasanifar A. Amphiphilic block co-polymers: Preparation and application in nanodrug and gene delivery. Acta Biomater. 2012;8(6):2017-33.

86. Andrade F, Videira M, Ferreira D, Sarmento B. Micelle-based systems for pulmonary drug delivery and targeting. Drug Delivery Letters. 2011;1(2):171-85.

87. Torchilin V. Micellar nanocarriers: pharmaceutical perspectives. Pharm Res. 2007;24(1):1-16.

88. Moghimi SM, Hunter AC. Poloxamers and poloxamines in nanoparticle engineering and experimental medicine. Trends Biotechnol. 2000;18(10):412-20.

89. Kabanov AV, Batrakova EV, Alakhov VY. Pluronic block copolymers as novel polymer therapeutics for drug and gene delivery. J Control Release. 2002;82(2-3):189-212.

90. Urbani CN, Bell CA, Lonsdale D, Whittaker MR, Monteiro MJ. Self-Assembly of amphiphilic polymeric dendrimers synthesized with selective degradable linkages Macromolecules. 2008;41:76-86.

91. Letchford K, Burt H. A review of the formation and classification of amphiphilic block copolymer nanoparticulate structures: micelles, nanospheres, nanocapsules and polymersomes. European Journal of Pharmaceutics and Biopharmaceutics. 2007;65(3):259-69.

92. Myers D. Surfactant science and technology. 3 ed. USA: Wiley-Interscience; 2006. 380 p.

93. Malmsten M. Surfactants and Polymers in Drug Delivery Marcel Dekker, Inc.; 2002. 335 p.

94. Babinot J, Guigner JM, Renard E, Langlois V. A micellization study of medium chain length poly(3-hydroxyalkanoate)-based amphiphilic diblock copolymers. J Colloid Interface Sci. 2012;375(1):88-93.

95. Letchford K, Liggins R, Burt H. Solubilization of hydrophobic drugs by methoxy poly(ethylene glycol)-block-polycaprolactone diblock copolymer micelles: theoretical and experimental data and correlations. J Pharm Sci. 2008;97(3):1179-90.

96. Sawant R, Jhaveri A. Micellar nanopreparations for medicine. In: Torchilin V, editor. Handbook of Nanobiomedical Research: Fundamentals, Applications and Recent Developments. Frontiers in Nanobiomedical Research. 3: World Scientific; 2014.

97. Study of Weekly GenexolÂ®-PM Plus Gemcitabine in Subjects With Recurrent and Metastatic Adenocarcinoma of the Pancreas 2016. Available from: https://clinicaltrials.gov/ct2/show/NCT02739633.

98. Evaluate the Efficacy and Safety of Genexol®-PM Compared to Genexol® in Recurrent or Metastatic Breast Cancer 2016. Available from: https://clinicaltrials.gov/ct2/show/NCT00876486.

99. A Trial to Evaluate Efficacy and Safety of the Combination Therapy of Genexol®-PM Plus Carboplatin® Compared to Genexol® Plus Carboplatin® as a Firstline Treatment in Subjects With Ovarian Cancer 2016. Available from: https://clinicaltrials.gov/ct2/show/NCT01276548.

100. A Phase II Trial of Genexol-PM and Gemcitabine in Patients With Advanced Non-small-cell Lung Cancer 2016. Available from: https://clinicaltrials.gov/ct2/show/NCT01770795.

101. A Clinical Trial of Paclitaxel Loaded Polymeric Micelle in Patients With Taxane-Pretreated Recurrent Breast Cancer 2016. Available from: https://clinicaltrials.gov/ct2/show/NCT00912639.

102. A Study of NK012 in Patients With Relapsed Small Cell Lung Cancer 2016. Available from: https://clinicaltrials.gov/ct2/show/NCT00951613.

103. A Study of NK012 in Patients With Advanced, Metastatic Triple Negative Breast Cancer 2016. Available from: https://clinicaltrials.gov/ct2/show/NCT00951054.

104. A Phase III Study of NK105 in Patients With Breast Cancer 2016. Available from: https://clinicaltrials.gov/ct2/show/NCT01644890.

105. Matsumura Y, Hamaguchi T, Ura T, Muro K, Yamada Y, Shimada Y, et al. Phase I clinical trial and pharmacokinetic evaluation of NK911, a micelle-encapsulated doxorubicin. Br J Cancer. 2004;91(10):1775-81.

106. A Phase 1 Dose-Escalation and Pharmacokinetic Study of NC-4016 in Patients With Advanced Solid Tumors or Lymphoma 2016. Available from: https://clinicaltrials.gov/ct2/show/NCT01999491.

107. Combination Therapy With NC-6004 and Gemcitabine Versus Gemcitabine Alone in Pancreatic Cancer 2016. Available from: https://clinicaltrials.gov/ct2/show/NCT02043288.

108. Combination Therapy With NC-6004 and Gemcitabine in Advanced Solid Tumors or Non-Small Cell Lung, Biliary and Bladder Cancer 2016. Available from: https://clinicaltrials.gov/ct2/show/NCT02240238.

109. A Study Using Intravenous Paxceedâ„¢ to Treat Patients With Rheumatoid Arthritis 2016. Available from: https://clinicaltrials.gov/ct2/show/NCT00055133.

110. Micellar Paclitaxel to Treat Severe Psoriasis 2016. Available from: https://clinicaltrials.gov/ct2/show/NCT00006276.

111. Efficacy Study of Maintenance IT-101 Therapy for Ovarian Cancer Patients 2016. Available from: https://clinicaltrials.gov/ct2/show/NCT00753740.

112. Study of CRLX101 (Formerly Named IT-101) in the Treatment of Advanced Solid Tumors 2016. Available from: https://clinicaltrials.gov/ct2/show/NCT00333502.

113. Effects of an Antioxidant-Enriched Multivitamin Supplement on Inflammation and Oxidative Stress in Cystic Fibrosis 2016. Available from: https://clinicaltrials.gov/ct2/show/NCT01859390.

114. Matsumura Y. Preclinical and clinical studies of NK012, an SN-38-incorporating polymeric micelles, which is designed based on EPR effect. Adv Drug Deliv Rev. 2011;63(3):184-92.

115. Kato K, Chin K, Yoshikawa T, Yamaguchi K, Tsuji Y, Esaki T, et al. Phase II study of NK105, a paclitaxel-incorporating micellar nanoparticle, for previously treated advanced or recurrent gastric cancer. Invest New Drugs. 2011.

116. Gilani K, Moazeni E, Ramezanli T, Amini M, Fazeli MR, Jamalifar H. Development of respirable nanomicelle carriers for delivery of amphotericin B by jet nebulization. J Pharm Sci. 2011;100(1):252-9.

117. Wang CH, Wang WT, Hsiue GH. Development of polyion complex micelles for encapsulating and delivering amphotericin B. Biomaterials. 2009;30(19):3352-8.

118. Kim DW, Kim SY, Kim HK, Kim SW, Shin SW, Kim JS, et al. Multicenter phase II trial of Genexol-PM, a novel Cremophor-free, polymeric micelle formulation of paclitaxel, with cisplatin in patients with advanced non-small-cell lung cancer. Ann Oncol. 2007;18(12):2009-14.

119. Lee KS, Chung HC, Im SA, Park YH, Kim CS, Kim SB, et al. Multicenter phase II trial of Genexol-PM, a Cremophor-free, polymeric micelle formulation of paclitaxel, in patients with metastatic breast cancer. Breast Cancer Res Treat. 2008;108(2):241-50.

120. Saif MW, Rubin MS, Figueroa JA, Kerr RO. Multicenter phase II trial of Genexol-PM (GPM), a novel Cremophor-free, polymeric micelle formulation of paclitaxel in patients with advanced pancreatic cancer (APC): Final results. . 2008 Gastrointestinal Cancers Symposium; Orlando2008.

121. Valle JW, Armstrong A, Newman C, Alakhov V, Pietrzynski G, Brewer J, et al. A phase 2 study of SP1049C, doxorubicin in P-glycoprotein-targeting pluronics, in patients with advanced adenocarcinoma of the esophagus and gastroesophageal junction. Invest New Drugs. 2011;29(5):1029-37.

122. Ho K, Li W, Wong C, Li P. Amphiphilic polymeric particles with core-shell nanostructures: emulsion-based syntheses and potential applications. Colloid and Polymer Science. 2010;288(16-17):1503-23.

123. Kwon GS. Amphiphilic block copolymer micelles for nanoscale drug delivery. DRUG DEVELOP RES. 2006;67:15-22.

124. Owens DE, Peppas NA. Opsonization, biodistribution, and pharmacokinetics of polymeric nanoparticles. Int J Pharm. 2006;307(1):93-102.

125. Moghimi SM, Szebeni J. Stealth liposomes and long circulating nanoparticles: critical issues in pharmacokinetics, opsonization and protein-binding properties. Prog Lipid Res. 2003;42(6):463-78.

126. Chaudhari KR, Ukawala M, Manjappa AS, Kumar A, Mundada PK, Mishra AK, et al. Opsonization, biodistribution, cellular uptake and apoptosis study of PEGylated PBCA nanoparticle as potential drug delivery carrier. Pharm Res. 2012;29(1):53-68.

127. Rösler A, Vandermeulen GW, Klok HA. Advanced drug delivery devices via self-assembly of amphiphilic block copolymers. Adv Drug Deliv Rev. 2001;53(1):95-108.

128. Kumar M, Kumar N, Domb A, Arora M. Pharmaceutical polymeric controlled drug delivery systems. Filled Elastomers Drug Delivery Systems. 2002;160:45-117.

129. Smith A, Xu X, McCormick C. Stimuli-responsive amphiphilic (co)polymers via RAFT polymerization. Progress in Polymer Science. 2010;35(1-2):45-93.

130. Ward MA, Georgiou TK. Thermoresponsive Polymers for Biomedical Applications. Polymers. 2011;3:1215-42.

131. Motornov M, Roiter Y, Tokarev I, Minko S. Stimuli-responsive nanoparticles, nanogels and capsules for integrated multifunctional intelligent systems. Progress in Polymer Science. 2010;35(1-2):174-211.

132. Hu Y, Litwin T, Nagaraja AR, Kwong B, Katz J, Watson N, et al. Cytosolic delivery of membrane-impermeable molecules in dendritic cells using pH-responsive core-shell nanoparticles. Nano Lett. 2007;7(10):3056-64.

133. Morishita M, Goto T, Nakamura K, Lowman AM, Takayama K, Peppas NA. Novel oral insulin delivery systems based on complexation polymer hydrogels: single and multiple administration studies in type 1 and 2 diabetic rats. J Control Release. 2006;110(3):587-94.

134. Kamei N, Morishita M, Chiba H, Kavimandan NJ, Peppas NA, Takayama K. Complexation hydrogels for intestinal delivery of interferon beta and calcitonin. J Control Release. 2009;134(2):98-102.

135. Guo W, Wang T, Tang X, Zhang Q, Yu F, Pei M. Triple stimuli-responsive amphiphilic glycopolymer

Journal of Polymer Science Part A: Polymer Chemistry Volume 52, Issue 15. Journal of Polymer Science Part A: Polymer Chemistry [Internet]. 2014 01; 52(15):[2131-8 pp.]. Available from: http://onlinelibrary.wiley.com/doi/10.1002/pola.27222/abstract.

136. Liu Y, Samsonova O, Sproat B, Merkel O, Kissel T. Biophysical characterization of hyper-branched polyethylenimine-graft-polycaprolactone-block-mono-methoxyl-poly(ethylene glycol) copolymers (hy-PEI-PCL-mPEG) for siRNA delivery. J Control Release. 2011;153(3):262-8.

137. Yang XZ, Dou S, Sun TM, Mao CQ, Wang HX, Wang J. Systemic delivery of siRNA with cationic lipid assisted PEG-PLA nanoparticles for cancer therapy. J Control Release. 2011;156(2):203-11.

138. Sun J, Luo T, Sheng R, Li H, Wang Z, Cao A. Intracellular plasmid DNA delivery by self-assembled nanoparticles of amphiphilic PHML-b-PLLA-b-PHML copolymers and the endocytosis pathway analysis. J Biomater Appl. 2016.

139. Murata N, Takashima Y, Toyoshima K, Yamamoto M, Okada H. Anti-tumor effects of anti-VEGF siRNA encapsulated with PLGA microspheres in mice. J Control Release. 2008;126(3):246-54.

140. Alshamsan A, Haddadi A, Hamdy S, Samuel J, El-Kadi AO, Uludağ H, et al. STAT3 Silencing in Dendritic Cells by siRNA Polyplexes Encapsulated in PLGA Nanoparticles for the Modulation of Anticancer Immune Response. Mol Pharm. 2010.

141. Mishra S, Peddada LY, Devore DI, Roth CM. Poly(alkylene oxide) Copolymers for Nucleic Acid Delivery. Acc Chem Res. 2012.

142. Kuo JH. Effect of Pluronic-block copolymers on the reduction of serum-mediated inhibition of gene transfer of polyethyleneimine-DNA complexes. Biotechnol Appl Biochem. 2003;37(Pt 3):267-71.

143. Wang M, Wu B, Lu P, Tucker JD, Milazi S, Shah SN, et al. Pluronic-PEI copolymers enhance exon-skipping of 2'-O-methyl phosphorothioate oligonucleotide in cell culture and dystrophic mdx mice. Gene Ther. 2014;21(1):52-9.

144. Davis ME. The first targeted delivery of siRNA in humans via a self-assembling, cyclodextrin polymer-based nanoparticle: from concept to clinic. Mol Pharm. 2009;6(3):659-68.

145. Zhu Y, Sheng R, Luo T, Li H, Sun W, Li Y, et al. Amphiphilic cationic [dendritic poly(L-lysine)]-block-poly(L-lactide)-block-[dendritic poly(L-lysine)]s in aqueous solution: self-aggregation and interaction with DNA as gene delivery carriers. Macromol Biosci. 2011;11(2):174-86.

146. Holzerny P, Ajdini B, Heusermann W, Bruno K, Schuleit M, Meinel L, et al. Biophysical properties of chitosan/siRNA polyplexes: profiling the polymer/siRNA interactions and bioactivity. J Control Release. 2012;157(2):297-304.

147. Shum VW, Gabrielson NP, Forrest ML, Pack DW. The effects of PVP(Fe(III)) catalyst on polymer molecular weight and gene delivery via biodegradable cross-linked polyethylenimine. Pharm Res. 2012;29(2):500-10.

148. Ulasov AV, Khramtsov YV, Trusov GA, Rosenkranz AA, Sverdlov ED, Sobolev AS. Properties of PEI-based polyplex nanoparticles that correlate with their transfection efficacy. Mol Ther. 2011;19(1):103-12.

149. Commander NJ, Brewer JM, Wren BW, Spencer SA, Macmillan AP, Stack JA. Liposomal delivery of p-ialB and p-omp25 DNA vaccines improves immunogenicity but fails to provide full protection against B. melitensis challenge. Genet Vaccines Ther. 2010;8:5.

150. Klausner EA, Zhang Z, Wong SP, Chapman RL, Volin MV, Harbottle RP. Corneal gene delivery: chitosan oligomer as a carrier of CpG rich, CpG free or S/MAR plasmid DNA. J Gene Med. 2012;14(2):100-8.

151. Rozema DB, Lewis DL, Wakefield DH, Wong SC, Klein JJ, Roesch PL, et al. Dynamic PolyConjugates for targeted in vivo delivery of siRNA to hepatocytes. Proc Natl Acad Sci U S A. 2007;104(32):12982-7.

152. Mudd SR, Trubetskoy VS, Blokhin AV, Weichert JP, Wolff JA. Hybrid PET/CT for noninvasive pharmacokinetic evaluation of dynamic PolyConjugates, a synthetic siRNA delivery system. Bioconjug Chem. 2010;21(7):1183-9.

153. Itaka K, Osada K, Morii K, Kim P, Yun SH, Kataoka K. Polyplex nanomicelle promotes hydrodynamic gene introduction to skeletal muscle. J Control Release. 2010;143(1):112-9.

154. Uchida S, Itaka K, Chen Q, Osada K, Miyata K, Ishii T, et al. Combination of chondroitin sulfate and polyplex micelles from Poly(ethylene glycol)-poly{N'-[N-(2-aminoethyl)-2-aminoethyl]aspartamide} block copolymer for prolonged in vivo gene transfection with reduced toxicity. J Control Release. 2011;155(2):296-302.

155. Chen Q, Osada K, Ishii T, Oba M, Uchida S, Tockary TA, et al. Homo-catiomer integration into PEGylated polyplex micelle from block-catiomer for systemic anti-angiogenic gene therapy for fibrotic pancreatic tumors. Biomaterials. 2012;33(18):4722-30.

156. Chen YC, Jiang LP, Liu NX, Ding L, Liu XL, Wang ZH, et al. Enhanced gene transduction into skeletal muscle of mice in vivo with pluronic block copolymers and ultrasound exposure. Cell Biochem Biophys. 2011;60(3):267-73.

157. Davis ME, Zuckerman JE, Choi CH, Seligson D, Tolcher A, Alabi CA, et al. Evidence of RNAi in humans from systemically administered siRNA via targeted nanoparticles. Nature. 2010;464(7291):1067-70.

158. Zuckerman JE, Hsueh T, Koya RC, Davis ME, Ribas A. siRNA knockdown of ribonucleotide reductase inhibits melanoma cell line proliferation alone or synergistically with temozolomide. J Invest Dermatol. 2011;131(2):453-60.

159. Phase I Single Dose-Ranging Study Of Formulated hIGF-1 Plasmid In Subjects With Cubital Tunnel Syndrome. . Available from: http://www.gemcris.od.nih.gov/Contents/GC_CLIN_TRIAL_RPT_VIEW.asp?WIN_TYPE=R&CTID=378.

160. Phase I/II Multi-Center, Open-Label, Multiple Administration Trial of the Safety, Tolerability, and Efficacy of an IFN-alpha/IL-12 Plasmid-Based Therapeutic. . Available from: http://www.gemcris.od.nih.gov/Contents/GC_CLIN_TRIAL_RPT_VIEW.asp?WIN_TYPE=R&CTID=116.

161. A Multicenter, Open-Label, Multiple Administration, Study of the Safety, Tolerability and Efficacy of IFN α/IL-12 Combination Gene Therapy in Patients with Squamous Cell Carcinoma of the Head and Neck (SCCHN). . Available from: http://www.gemcris.od.nih.gov/Contents/GC_CLIN_TRIAL_RPT_VIEW.asp?WIN_TYPE=R&CTID=184.

162. Oh Y-K, Park TG. siRNA delivery systems for cancer treatment. Advanced Drug Delivery Reviews. 2009;61(10):850-62.

163. Aliabadi HM, Landry B, Sun C, Tang T, Uludağ H. Supramolecular assemblies in functional siRNA delivery: Where do we stand? Biomaterials. 2012;33(8):2546-69.

164. Ma B, Zhang S, Jiang H, Zhao B, Lv H. Lipoplex morphologies and their influences on transfection efficiency in gene delivery. J Control Release. 2007;123(3):184-94.

165. Esposito C, Generosi J, Mossa G, Masotti A, Castellano AC. The analysis of serum effects on structure, size and toxicity of DDAB-DOPE and DC-Chol-DOPE lipoplexes contributes to explain their different transfection efficiency. Colloids Surf B Biointerfaces. 2006;53(2):187-92.

166. Masotti A, Mossa G, Cametti C, Ortaggi G, Bianco A, Grosso ND, et al. Comparison of different commercially available cationic liposome-DNA lipoplexes: Parameters influencing toxicity and transfection efficiency. Colloids Surf B Biointerfaces. 2009;68(2):136-44.

167. Kenny GD, Kamaly N, Kalber TL, Brody LP, Sahuri M, Shamsaei E, et al. Novel multifunctional nanoparticle mediates siRNA tumour delivery, visualisation and therapeutic tumour reduction in vivo. J Control Release. 2011;149(2):111-6.

168. Akinc A, Goldberg M, Qin J, Dorkin JR, Gamba-Vitalo C, Maier M, et al. Development of lipidoid-siRNA formulations for systemic delivery to the liver. Mol Ther. 2009;17(5):872-9.

169. Rejman J, Conese M, Hoekstra D. Gene transfer by means of lipo- and polyplexes: role of clathrin and caveolae-mediated endocytosis. J Liposome Res. 2006;16(3):237-47.

170. Wong AW, Scales SJ, Reilly DE. DNA internalized via caveolae requires microtubule-dependent, Rab7-independent transport to the late endocytic pathway for delivery to the nucleus. J Biol Chem. 2007;282(31):22953-63.

171. Lu JJ, Langer R, Chen J. A novel mechanism is involved in cationic lipid-mediated functional siRNA delivery. Mol Pharm. 2009;6(3):763-71.

172. Kapoor M, Burgess DJ, Patil SD. Physicochemical characterization techniques for lipid based delivery systems for siRNA. Int J Pharm. 2012;427(1):35-57.

173. Mignet N, Richard C, Seguin J, Largeau C, Bessodes M, Scherman D. Anionic pH-sensitive pegylated lipoplexes to deliver DNA to tumors. Int J Pharm. 2008;361(1-2):194-201.

174. Srinivasan C, Burgess DJ. Optimization and characterization of anionic lipoplexes for gene delivery. J Control Release. 2009;136(1):62-70.

175. Kapoor M, Burgess DJ. Efficient and safe delivery of siRNA using anionic lipids: Formulation optimization studies. Int J Pharm. 2012;432(1-2):80-90.

176. Kurreck J. RNA Interference: From Basic Research to Therapeutic Applications. Angewandte Chemie International Edition. 2009;48(8):1378-98.

177. Petros RA, DeSimone JM. Strategies in the design of nanoparticles for therapeutic applications. Nat Rev Drug Discov. 2010;9(8):615-27.

178. Rossi JJ. RNAi therapeutics: SNALPing siRNAs in vivo. Gene Therapy. 2005;13(7):583-4.

179. Morrissey DV, Blanchard K, Shaw L, Jensen K, Lockridge JA, Dickinson B, et al. Activity of stabilized short interfering RNA in a mouse model of hepatitis B virus replication. Hepatology. 2005;41(6):1349-56.

180. Semple SC, Akinc A, Chen J, Sandhu AP, Mui BL, Cho CK, et al. Rational design of cationic lipids for siRNA delivery. Nature Biotechnology. 2010;28(2):172-6.

181. Huang L, Liu Y. In Vivo Delivery of RNAi with Lipid-Based Nanoparticles. Annual Review of Biomedical Engineering. 2011;13(1):507-30.

182. Nguyen T, Menocal EM, Harborth J, Fruehauf JH. RNAi therapeutics: An update on delivery. Current Opinion in Molecular Therapeutics. 2008;10(2):158-67.

183. Ciani L, Casini A, Gabbiani C, Ristori S, Messori L, Martini G. DOTAP/DOPE and DC-Chol/DOPE lipoplexes for gene delivery studied by circular dichroism and other biophysical techniques. Biophys Chem. 2007;127(3):213-20.

184. Zhang Y, Li H, Sun J, Gao J, Liu W, Li B, et al. DC-Chol/DOPE cationic liposomes: a comparative study of the influence factors on plasmid pDNA and siRNA gene delivery. Int J Pharm. 2010;390(2):198-207.

185. Constien Rea, inventorNovel Lipids and compositions for intracellular delivery of biologically active compounds2012.

186. Simon A. Phase I Safety, Pharmacokinetic and Pharmacodynamic Results for ALN-PCS - Preliminary Study Results. In: Pharmaceuticals A, editor. 2012.

187. Semple SC, Klimuk SK, Harasym TO, Santos ND, Ansell SM, Wong KF, et al. Efficient encapsulation of antisense oligonucleotides in lipid vesicles using ionizable aminolipids: formation of novel small multilamellar vesicle structures. Biochimica et Biophysica Acta. 2001;1510:152-66.

188. Aliabadi HM, Landry B, Mahdipoor P, Hsu CY, Uludag H. Effective down-regulation of breast cancer resistance protein (BCRP) by siRNA delivery using lipid-substituted aliphatic polymers. Eur J Pharm Biopharm. 2012;81(1):33-42.

189. Köhler E, Duberow-Drewe M, Drewe J, Ribes G, Loubatiéres-Mariani M, Mazer N, et al. Absorption of an aqueous solution of a new synthetic somatostatin analogue administered to man by gavage. Eur J Clin Pharmacol. 1987;33(2):167-71.

190. Xue HY, Wong HL. Tailoring nanostructured solid-lipid carriers for time-controlled intracellular siRNA kinetics to sustain RNAi-mediated chemosensitization. Biomaterials. 2011;32(10):2662-72.

191. Bondi ML, Azzolina A, Craparo EF, Lampiasi N, Capuano G, Giammona G, et al. Novel cationic solid-lipid nanoparticles as non-viral vectors for gene delivery. J Drug Target. 2007;15(4):295-301.

192. del Pozo-Rodríguez A, Delgado D, Solinís MA, Pedraz JL, Echevarría E, Rodríguez JM, et al. Solid lipid nanoparticles as potential tools for gene therapy: in vivo protein expression after intravenous administration. Int J Pharm. 2010;385(1-2):157-62.

193. Lobovkina T, Jacobson GB, Gonzalez-Gonzalez E, Hickerson RP, Leake D, Kaspar RL, et al. In vivo sustained release of siRNA from solid lipid nanoparticles. ACS Nano. 2011;5(12):9977-83.

194. Yu YH, Kim E, Park DE, Shim G, Lee S, Kim YB, et al. Cationic solid lipid nanoparticles for co-delivery of paclitaxel and siRNA. Eur J Pharm Biopharm. 2012;80(2):268-73.

195. Metwally AA, Blagbrough IS, Mantell JM. Quantitative Silencing of EGFP Reporter Gene by Self-Assembled siRNA Lipoplexes of LinOS and Cholesterol. Mol Pharm. 2012;9(11):3384-95.

196. Tagami T, Suzuki T, Matsunaga M, Nakamura K, Moriyoshi N, Ishida T, et al. Anti-angiogenic therapy via cationic liposome-mediated systemic siRNA delivery. Int J Pharm. 2012;422(1-2):280-9.

197. Zhou S, Kawakami S, Higuchi Y, Yamashita F, Hashida M. The involvement of NK cell activation following intranasal administration of CpG DNA lipoplex in the prevention of pulmonary metastasis and peritoneal dissemination in mice. Clin Exp Metastasis. 2012;29(1):63-70.

198. Wu SY, Chang HI, Burgess M, McMillan NA. Vaginal delivery of siRNA using a novel PEGylated lipoplex-entrapped alginate scaffold system. J Control Release. 2011;155(3):418-26.

199. Rothdiener M, Muller D, Castro PG, Scholz A, Schwemmlein M, Fey G, et al. Targeted delivery of SiRNA to CD33-positive tumor cells with liposomal carrier systems. J Control Release. 2010;144(2):251-8.

200. Aleku M, Schulz P, Keil O, Santel A, Schaeper U, Dieckhoff B, et al. Atu027, a liposomal small interfering RNA formulation targeting protein kinase N3, inhibits cancer progression. Cancer Res. 2008;68(23):9788-98.

201. Atu027 Plus Gemcitabine in Advanced or Metastatic Pancreatic Cancer (Atu027-I-02) 2016. Available from: https://clinicaltrials.gov/ct2/show/NCT01808638.

202. Phase I Pilot Study of Gene Therapy for Cystic Fibrosis Using Cationic Liposome Mediated Gene Transfer . Available from: http://www.clinicaltrials.gov/ct2/show/NCT00004471.

203. Phase I Study of IV DOTAP: Cholesterol-Fus1 in Non-Small-Cell Lung Cancer . Available from: http://clinicaltrials.gov/show/NCT00059605.

204. FUS1-nanoparticles and Erlotinib in Stage IV Lung Cancer 2016. Available from: https://clinicaltrials.gov/ct2/show/NCT01455389.

205. A Study to Determine Safety, Pharmacokinetics and Pharmacodynamics of Intravenous TKM 080301 in Neuroendocrine Tumors (NET) and Adrenocortical Carcinoma (ACC) Patients 2016. Available from: https://clinicaltrials.gov/ct2/show/NCT01262235.

206. TKM 080301 for Primary or Secondary Liver Cancer 2016. Available from: https://clinicaltrials.gov/ct2/show/NCT01437007.

207. QPI-1002 for Prevention of Delayed Graft Function in Recipients of an Older Donor Kidney Transplant (ReGIFT) https://clinicaltrials.gov/ct2/show/NCT02610296 [

208. A Study to Evaluate Efficacy and Safety of QPI-1002 for Prevention of Acute Kidney Injury Following Cardiac Surgery https://www.clinicaltrials.gov/ct2/show/NCT02610283 [

209. PF-04523655 Dose Escalation Study, and Evaluation of PF-04523655 With/Without Ranibizumab in Diabetic Macular Edema (DME) (MATISSE) https://www.clinicaltrials.gov/ct2/show/NCT01445899 [

210. Perche F, Lambert O, Berchel M, Jaffrès P-A, Pichon C, Midoux P. Gene transfer by histidylated lipopolyplexes: A dehydration method allowing preservation of their physicochemical parameters and transfection efficiency. International Journal of Pharmaceutics. 2012;423:144-50.

211. Whitmore M, Li S, Huang L. LPD lipopolyplex initiates a potent cytokine response and inhibits tumor growth. Gene Therapy. 1999;6:1867-75.

212. Matsuura M, Yamazaki Y, Sugiyama M, Kondo M, Ori H, Nango M, et al. Polycation liposome-mediated gene transfer in vivo. Biochimica et Biophysica Acta (BBA) - Biomembranes. 2003;1612(2):136-43.

213. Urbiola K, Garcia L, Zalba S, Garrido MJ, Tros de Ilarduya C. Efficient serum-resistant lipopolyplexes targeted to the folate receptor. European journal of pharmaceutics and biopharmaceutics : official journal of Arbeitsgemeinschaft fur Pharmazeutische Verfahrenstechnik eV. 2013;83(3):358-63.

214. Pelisek J, Gaedtke L, DeRouchey J, Walker GF, Nikol S, Wagner E. Optimized lipopolyplex formulations for gene transfer to human colon carcinoma cells under in vitro conditions. The Journal of Gene Medicine. 2006;8(2):186-97.

215. Gao X, Huang L. Potentiation of cationic liposome-mediated gene delivery by polycations. Biochemistry. 1996;35(3):1027-36.

216. Garcia L, Bunuales M, Duzgunes N, Ilarduya CTd. Serum-resistant lipopolyplexes for gene delivery to liver tumour cells. European Journal of Pharmaceutics and Biopharmaceutics. 2007;67:58-66.

217. Vangasseri DP, Han SJ, Huang L. Lipid-protamine-DNA-mediated antigen delivery. Curr Drug Deliv. 2005;2(4):401-6.

218. Weide B, Garbe C, Rammensee H-G, Pascolo S. Plasmid DNA- and messenger RNA-based anti-cancer vaccination. Immunology Letters. 2008;115(1):33-42.

219. Li SD, Huang L. Targeted delivery of antisense oligodeoxynucleotide and small interference RNA into lung cancer cells. Mol Pharm. 2006a;3(5):579-88.

220. Li SD, Huang L. Surface-modified LPD nanoparticles for tumor targeting. Ann N Y Acad Sci. 2006b:1-8.

221. Hoerr I, Obst R, Rammensee HG, Jung G. In vivo application of RNA leads to induction of specific cytotoxic T lymphocytes and antibodies. Eur J Immunol. 2000;30(1):1-7.

222. Mockey M, Bourseau E, Chandrashekhar V, Chaudhuri A, Lafosse S, Le Cam E, et al. mRNA-based cancer vaccine: prevention of B16 melanoma progression and metastasis by systemic injection of MART1 mRNA histidylated lipopolyplexes. Cancer Gene Ther. 2007;14(9):802-14.

223. Birchall JC, Kellaway IW, Gumbleton M. Physical stability and in-vitro gene expression efficiency of nebulised lipid-peptide-DNA complexes. Int J Pharm. 2000;197(1-2):221-31.

224. Rehman Zu, Hoekstra D, Zuhorn IS. Kinases in cationic lipid/polymer-mediated gene delivery. Drug Discovery Today. 2010;15(23–24):1110.

225. Ramezani M, Khoshhamdam M, Dehshahri A, Malaekeh-Nikouei B. The influence of size, lipid composition and bilayer fluidity of cationic liposomes on the transfection efficiency of nanolipoplexes. Colloids Surf B Biointerfaces. 2009;72(1):1-5.

226. Garcia L, Urbiola K, Duzgunes N, Tros de Ilarduya C. Lipopolyplexes as nanomedicines for therapeutic gene delivery. Methods Enzymol. 2012;509:327-38.

227. Kurosaki T, Kishikawa R, Matsumoto M, Kodama Y, Hamamoto T, To H, et al. Pulmonary gene delivery of hybrid vector, lipopolyplex containing N-lauroylsarcosine, via the systemic route. J Control Release. 2009;136(3):213-9.

228. Alshamsan A, Hamdy S, Haddadi A, Samuel J, El-Kadi AOS, Uludağ H, et al. STAT3 Knockdown in B16 Melanoma by siRNA Lipopolyplexes Induces Bystander Immune Response In Vitro and In Vivo. Translational Oncology. 2011;4(3):178-88.

229. Li S, Huang L. In vivo gene transfer via intravenous administration of cationic lipid-protamine-DNA (LPD) complexes. Gene Ther. 1997;4(9):891-900.

230. Kudsiova L, Fridrich B, Ho J, Mustapa MF, Campbell F, Welser K, et al. Lipopolyplex ternary delivery systems incorporating C14 glycerol-based lipids. Mol Pharm. 2011;8(5):1831-47.

231. Welser K, Campbell F, Kudsiova L, Mohammadi A, Dawson N, Hart SL, et al. Gene Delivery Using Ternary Lipopolyplexes Incorporating Branched Cationic Peptides: The Role of Peptide Sequence and Branching. Mol Pharm. 2012;4:4.

232. Yan J, Berezhnoy NV, Korolev N, Su CJ, Nordenskiold L. Structure and internal organization of overcharged cationic-lipid/peptide/DNA self-assembly complexes. Biochim Biophys Acta. 2012;6(7):1794-800.

233. Junghans M, Loitsch SM, Steiniger SC, Kreuter J, Zimmer A. Cationic lipid-protamine-DNA (LPD) complexes for delivery of antisense c-myc oligonucleotides. Eur J Pharm Biopharm. 2005;60(2):287-94.

234. Tseng YC, Mozumdar S, Huang L. Lipid-based systemic delivery of siRNA. Advanced drug delivery reviews. 2009;61(9):721-31.

235. Sun P, Zhong M, Shi X, Li Z. Anionic LPD complexes for gene delivery to macrophage: preparation, characterization and transfection in vitro. J Drug Target. 2008;16(9):668-78.

236. Hu Y, Li K, Wang L, Yin S, Zhang Z, Zhang Y. Pegylated immuno-lipopolyplexes: A novel non-viral gene delivery system for liver cancer therapy. Journal of Controlled Release. 2010;144:75-81.

237. Hu Y, Shen Y, Ji B, Wang L, Zhang Z, Zhang Y. Combinational RNAi gene therapy of hepatocellular carcinoma by targeting human EGFR and TERT. European Journal of Pharmaceutical Sciences. 2011a;42:387-91.

238. Hu Y, Shen Y, Ji B, Yin S, Ren X, Chen T, et al. Liver-specific gene therapy of hepatocellular carcinoma by targeting human telomerase reverse transcriptase with pegylated immuno-lipopolyplexes. European Journal of Pharmaceutics and Biopharmaceutics. 2011b;78:320-5.

239. Perche F, Benvegnu T, Berchel M, Lebegue L, Pichon C, Jaffrès P-A, et al. Enhancement of dendritic cells transfection in vivo and of vaccination against B16F10 melanoma with mannosylated histidylated lipopolyplexes loaded with tumor antigen messenger RNA. Nanomedicine: Nanotechnology, Biology, and Medicine. 2011;7:445-53.

240. Dileo J, Banerjee R, Whitmore M, Nayak JV, Falo LD, Jr., Huang L. Lipid-protamine-DNA-mediated antigen delivery to antigen-presenting cells results in enhanced anti-tumor immune responses. Mol Ther. 2003;7(5 Pt 1):640-8.

241. Whitmore MM, Li S, Falo L, Jr., Huang L. Systemic administration of LPD prepared with CpG oligonucleotides inhibits the growth of established pulmonary metastases by stimulating innate and acquired antitumor immune responses. Cancer Immunol Immunother. 2001;50(10):503-14.

242. A Phase I/II Study of Intraperitoneal EGEN-001 . Available from: http://www.gemcris.od.nih.gov/ .

243. Study of Safety & Biological Activity of IP GEN-1 With Neoadjuvant Chemo in Ovarian Cancer 2016. Available from: https://clinicaltrials.gov/ct2/show/NCT02480374.

244. EGEN-001 in Treating Patients With Persistent or Recurrent Ovarian Epithelial Cancer, Fallopian Tube Cancer, or Primary Peritoneal Cancer 2016. Available from: https://clinicaltrials.gov/ct2/show/NCT01118052.

245. A Study of the Safety and Biological Activity of Intraperitoneal (IP) EGEN-001 Administered Alone and in Combination With Standard Chemotherapy in Colorectal Peritoneal Carcinomatosis Patients 2016. Available from: https://clinicaltrials.gov/ct2/show/NCT01300858.

CHAPTER 3

Formulation Studies and Efficacy Assessment of Different siRNA Delivery Systems

The information presented in this chapter was partially published in the following publication:

1) D Rafael, P Gener, F Andrade, J Seras, S Montero, Y Fernandez, I Abasolo, S Schwartz Jr., M Videira, Functional Validation of Amphiphilic-Based Polymeric Micelles for siRNA Delivery and Cancer Stem Cells Genes Inhibition, *Article in preparation*.

Table of contents

3.1. Introduction

Different types of non-viral systems for siRNA delivery have been proposed in the last years, many of them based on cationic polymers or polypeptides such as PEI, CS, PLL, among others.

As previously referred, CS is a natural cationic polysaccharide composed of glucosamine and N-acetyl glucosamine, whose ability to interact with the negatively charged OGN is related, among other factors, with its MW and DD. Usually a higher DD enables a better interaction with the genetic material and, while higher MW CS promotes a better stability of the complexes, low MW ones leads to a better intracellular OGN release (1-3). Therefore, CS MW should be regulated in order to achieve a balance between protection and release of the OGN. To improve the transfection efficiency of CS-based carriers different chemical modifications, such as the combination of CS with dextran, N-dodecyl, glycyrrhizin, folic acid or even with PEI have been investigated (4-10). Additionally, the use of the water-soluble salt forms of CS (chloride, acetate, aspartate, glutamate or lactate salts) is another possible strategy to improve the transfection efficiency (1, 2).

Regarding PEI, despite its high transfection efficiencies, by itself cannot be used as a DDS since presents some drawbacks mainly related with its high cationic charge density (11-13). The transfection efficiency and cytotoxicity of these polymers are highly dependent on their linear versus branched structure, the branching degree or their MW (12, 14-17). Usually PEI branched forms present higher transfection efficiencies than the linear forms. Furthermore, higher PEI MW are usually associated with higher buffering capacity, higher transfections efficiencies but also increased cytotoxicity. The adequate N/P ratio should be optimized for each formulation in order to find an equilibrium of charges to achieve maximum silencing efficacy with minor toxicity (12, 14, 16, 18). In order to improve PEI transfection efficiency by reducing cytotoxicity, avoid aggregation and decrease nonspecific interactions, many different strategies have been explored being PEI grafting with PEG one of the most common ones (19, 20).

With the objective of develop an innovative and efficient nanosystem for the delivery of siRNA, different polymeric-based formulations were designed until reach the optimal formulation proposed in this study, consisting in the combination of PEI and amphiphilic polymers. Thereby, we were able to reduce the PEI-related toxicity and improve the biological efficacy of the PEI-siRNA complexes.

The poloxamers (Pluronic®) are polymers consisting of ethylene oxide (EO) and propylene oxide (PO) chains arranged in a triblock structure (EOa-POb-EOa) (**Figure 3.1**) (21, 22). They were chosen to be included in formulation due to their recognized ability to enhance

the transfection efficiency of genetic material, and their PEGylated surface, offering stealth properties to the system (23). In order to assess the best polymer features, different types of Pluronic® were tested (**Table 3.1**).

Table 3. 1. Poly(ethylene glycol) (a) and polypropylene oxide (b) units, molecular weight (MW) and critical micelle concentration (CMC) values of the different Pluronic® used (according to the manufacturer).

Type of Pluronic®	Number of a units	Number of b units	Average MW of the polymer (g/mol)	CMC (M)	Approximate MW of PEG chains (g/mol)
F68	80	27	7,680 – 9,510	$4.8x10^{-4}$	4000
F108	141	44	12,700 – 17,400	$2.2x10^{-5}$	7000
F127	101	56	9,840 – 14,600	$2.8x10^{-6}$	5000

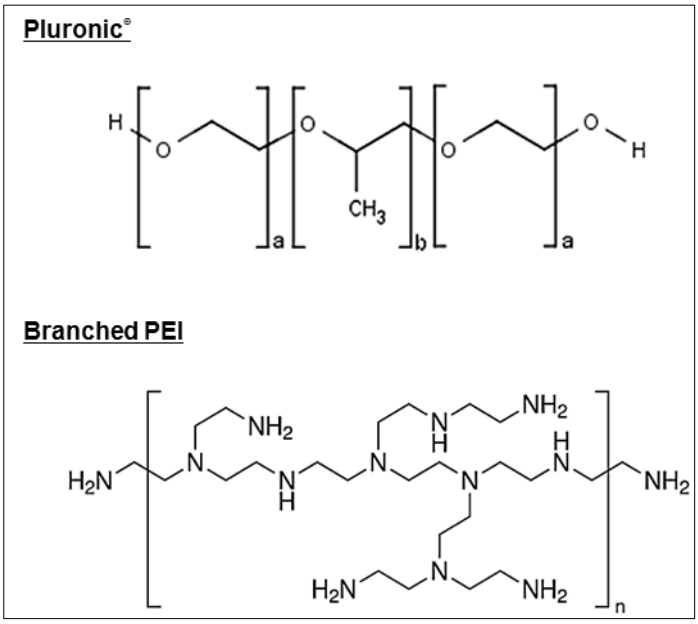


Figure 3. 1. General structure of Pluronic® and PEI polymers.

3.2. Materials and Methods

3.2.1. Materials

Different types of CS with the DD of ~86% (Protasan Ultrapure) were gently provided by NovaMatriX (USA), namely, glutamate-CS low (G113 - 160 kDa) and high (G213 - 470 kDa) MW, and hydrochloride-CS low (CL113 - 110 kDa) and high (CL213 - 270 kDa) MW (22,

24-26). Glycol-CS was purchased from Sigma Aldrich (Madrid, Spain). Pluronic® F68, F108 and F127 were kindly provided by BASF (Ludwigshafen, Germany), while 10k branched and 25k branched PEI were provided by Alfa Aesar (Thermo Fisher GmbH, Karlsruhe, Germany) and Sigma-Aldrich (Madrid, Spain), respectively.

siRNA against GFP (GFP-siRNA) and the scramble sequence (siC) were provided by two companies, Shangai Gene Pharma (China) and LifeTechnologies (Spain). siRNA-AlexaFluor 488 was brought from GE Healthcare Europe GmbH (Barcelona, Spain).

RXO-C colon cancer cells expressing GFP were generously provided by Dr. Diego Arango from CIBBIM-Nanomedicine. Breast cancer cell lines MDA-MB-231 (ATCC number HTB-26) and MCF7 (ATCC number HTB-22) were obtained from American Type Culture Collection (ATCC, LGC Standards, Barcelona, Spain). RPMI medium, phosphate buffered saline (PBS), and fetal bovine serum (FBS) were purchase from Lonza (Barcelona, Spain). Penicillin-streptomycin, L-glutamine, non-essential amino acids, sodium pyruvate, 0.25% Trypsin-EDTA, Lipofectamine® 2000, 4′,6-diamidino-2-phenylindole (DAPI), and LysoTracker® Red are brought from Life Technologies Ltd. (Madrid, Spain).

Other used reagents are methanol, ethanol, dimethyl sulfoxide (DMSO), 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), gelatin, paraformaldehyde, Triton X-100, and 5-([4,6-dichlorotriazin-2-yl]amino)fluorescein hydrochloride (5-DTAF) from Sigma-Aldrich (Madrid, Spain), and Type 1 ultrapure water (18.2 MΩ.cm at 25 ºC, Milli-Q®, Billerica, MA, USA).

3.2.2. Polyplexes Preparation

Polymer-siRNA complexes were prepared by simple complexation, adding the polymer solution dropwise to an equal volume of siRNA solution and quickly vortexed during few seconds before incubating them at room temperature for 30 minutes.

The CS-siRNA complexes were prepared at different N/P ratios calculated according to **Equation 3.1** and at different conditions **(Table 3.2)**.

$$\frac{N}{P}ratio = \frac{\frac{Mass\ of\ CS}{Mass\ per\ charge\ of\ CS\ \left(=160\frac{g}{mol}\right)}}{\frac{Mass\ of\ siRNA}{Mass\ per\ charge\ of\ phosphate\ \left(=330\frac{g}{mol}\right)}} \qquad \textbf{Equation 3.1}$$

For the PEI-based polyplexes, two types of branched PEI, namely 10k and 25k, were used. Different N/P ratios were tested ranging from 5 to 75 (**Table 3.4**) and calculated according to **Equation 3.2**.

$$\frac{N}{P}ratio = \frac{\frac{Mass\ of\ PEI}{Mass\ per\ charge\ of\ PEI\ \left(=43\frac{g}{mol}\right)}}{\frac{Mass\ of\ siRNA}{Mass\ per\ charge\ of\ phosphate\ \left(=330\frac{g}{mol}\right)}} \qquad \textbf{Equation 3.2}$$

3.2.3. PEI- and CS-based Micelles Preparation

Micelles were prepared using the thin-film hydration (FH) technique. Briefly, each amphiphilic polymer was individually weighted and dissolved in a mixture of methanol:ethanol (1:1). This mixture of solvents was chosen because the polymers are insoluble in ethanol alone (Class 3 solvent), but soluble in methanol (Class 2 solvent). By mixing both solvents was possible to solubilize the polymers and reduce the use of methanol, as previously described (27, 28). Then, the solvent was removed under vacuum and the formed film was left to dry overnight at room temperature to eliminate any remaining solvent. Afterwards, the film was hydrated with PBS for empty micelles or with the previously prepared polymer-siRNA polyplexes dispersion and vortexed during 1 minute. The obtained dispersion was filtered through a 0.22 µm syringe filter to remove possible aggregates.
In order to compare different preparation methods, PEI-based polyplexes containing micelles were also prepared by the direct dissolution method (DM). For that, the amphiphilic polymer was dissolved in an aqueous solution and added to the polyplexes solution. After vortexing, the mixture was left to incubate for 30 minutes and filtered through a 0.22 µm syringe filter.

3.2.4. Association Efficiency (AE)

The non-associated siRNA present in the aqueous phase of the polyplexes was separated by centrifugation with filtration (10,000 rpm, 10 minutes) using centrifugal devices with a 100K membrane (Nanosep® Centrifugal Devices, Millipore, USA) and measured by a spectrophotometry method (Nanodrop NP-1000, Thermo Scientific, USA). The association efficiency was calculated according to **Equation 3.3**. The polyplexes association efficiency was also assessed by agarose gel electrophoresis.

$$AE = \frac{\text{total amount of siRNA} - \text{free siRNA in filtrate}}{\text{total amount of siRNA}} \times 100 \qquad \text{Equation 3.3}$$

3.2.5. Particles Physicochemical Properties Characterization

Particles mean hydrodynamic diameter (md) and polydispersity index (Pdi) were measured by dynamic light scattering (DLS) and zeta potential was assessed by laser doppler micro-electrophoresis using a NanoZS (Malvern Instruments, UK). For each formulation, at least three batches were produced and analyzed.
Particle shape and morphology were observed by transmission electron microscopy (TEM) and Cryo-scanning electron microscopy (CryoSEM) (external services from IBMC and CEMUP, University of Porto, Portugal respectively).

3.2.6. Serum Stability

To assess the stability of formulations in the presence of serum the particles were incubated in a proportion of 1:1 with 50 % FBS and the mean diameter measured by DLS at different time-points: 0, 6, 12 and 24 hours.

3.2.7. Cell lines Culture Conditions

MDA-MB-231, MCF7 and RXO-C cells were cultured in RPMI medium supplemented with 10% FBS, 1% penicillin-streptomycin, 1% L-glutamine, 1% non-essential amino acids, and 1% of sodium pyruvate and maintained at 37 ^{0} C under a 5% CO_2 water saturated atmosphere. The medium was changed every other day and, upon confluence, cells were harvested from plates with a 0.25% trypsin-EDTA solution and passed to continue the expansion.

3.2.8. GFP Reporter Gene Silencing Assay

RXO-C colon cancer cells expressing GFP were used to assess the systems silencing efficacy. Polyplexes and micelles prepared using a GFP-siRNA and different cationic

polymers were added to cells seeded at a concentration of 5 x 10^4 cells/ml in 96 well plates. As positive controls were used complexes formed between Lipofectamine® 2000 and the GFP-siRNA prepared accordingly to the supplier instructions but at the same concentration of siRNA used for the tested polyplexes. On the other hand, cells transfected at the same conditions with polyplexes prepared with a siRNA of a random sequence (siC) were used as negative control. The expression of GFP in cells after transfection was assessed with a fluorescence microscope (Olympus, USA). The intensity of cells fluorescence was also measured using a Fluorescent Microplate reader FLX800 (BioTek, Germany).

3.2.9. *In vitro* Cytotoxicity Assay

The cytotoxicity of different components in MDA-MB-231 cells was assessed using the MTT assay. 5000 cells/well/100µl were seeded in 96-well plates and left to attach for 24 hours. Then, cells were incubated in the presence or absence of increasing concentrations of polymers/formulations for 24 hours. After this incubation time, the medium was changed and the cells left for 72 hours. Complete medium was used as negative control and 10% DMSO as positive control of toxicity. After the 72 hours incubation, 0.5 mg/ml of MTT was added to each well. The plates were incubated for an additional 4 hours at 37ºC, the medium discarded, and the formazan crystals produced by mitochondrial succinate dehydrogenase dissolved with DMSO. The absorbance of each well was read on a microplate reader (ELx800 absorbance reader, BioTek, Germany) at 590 nm and the cell viability calculated. The results of cell viability were used for the determination of IC_{50} index by nonlinear regression of the concentration-effect curve fit using Prism 6.02 software (GraphPad Software, Inc., CA, USA).

3.2.10. Conjugation of F127 with 5-DTAF

F127 was fluorescently conjugated with 5-DTAF in an aqueous medium via nucleophilic aromatic substitution by an addition-elimination pathway as previously described **(Figure 3.2)** (29).

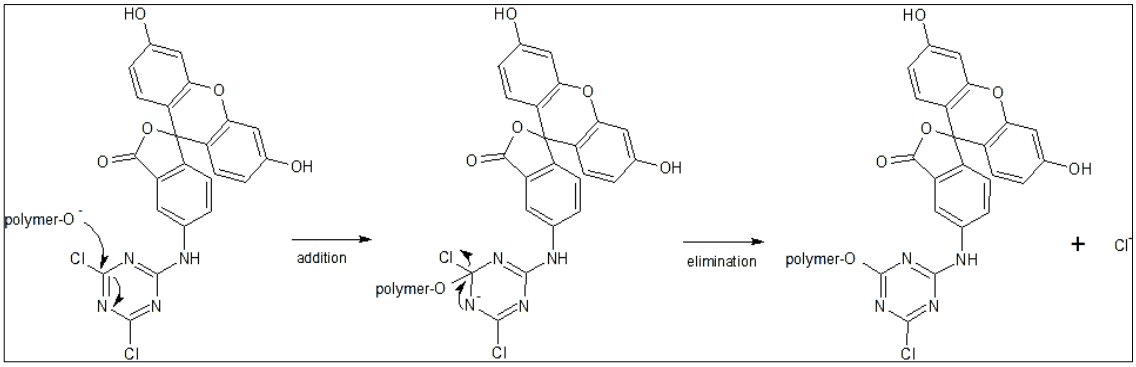


Figure 3. 2. Reaction schematic for the conjugation of F127 with 5-DTAF via nucleophilic aromatic substitution by an addition-elimination mechanism. At basic pH (9.3), the terminal hydroxyl group of PEG blocks presented in F127, attack the reactive moiety (2-amino-4,6-dichloro-s-triazine) on the 5-DTAF molecule, promoted by strong electron-withdrawing groups (N) of the s-triazine ring.

Briefly, a stock solution of 20 g/L 5-DTAF in DMSO was diluted in 0.1M sodium bicarbonate (pH 9.3) and added to a 6 % (w/v) F127 solution in 0.1M sodium bicarbonate (pH 9.3) to a final molar ratio of 1:2 (F127:5-DTAF). The reaction proceeded overnight in the dark at room temperature. The labeled polymer was purified from the excess of unreacted 5-DTAF by dialysis (12,000-14,000 MWCO Spectra/Por® membrane from Spectrum Europe BV, The Netherlands) against Type I ultrapure water. The dialyzed polymer solutions were lyophilized and stored in closed containers protected from light.

3.2.11. Internalization Assays

Flow cytometry and confocal microscopy were used to assess quantitatively and qualitatively the nanosystem internalization capacity in MDA-MB-231 breast cancer cells.

For the quantitative Fluorescence-activated cell sorting (FACS) assays, $2x10^5$ cells were seeded in 6 well plates and incubated for 24 hours. After this time, 5-DTAF-labelled micelles were added to cells and incubated during 2 and 4 hours after which the cells were washed with 1x PBS, detached with 0.25% trypsin-EDTA, resuspended in PBS supplemented with 10% FBS and DAPI (1 µg/mL) used for vital staining. The plate was analyzed in a cytometer Fortessa (BD Biosciences, USA). Data was analyzed with FCS Express 4 Flow Research Edition software (De Novo Software, Los Angeles, USA). Contaminants were removed by forward and side scatter gating. For each sample, at least 10000 individual cells were collected and the mean fluorescence intensity was evaluated.

For the qualitative confocal microscopy assay (Spectral Confocal Microscope MFV1000 Olympus, USA), cells were cultured in 0.1% gelatin-treated coverslips at a density of $2.5x10^5$ cells per well in 6 well plates. After 24 hours, the PM containing siRNA-AlexaFluor

488 were added to cells and incubated during 4 hours and further incubated for 30 minutes with the LysoTracker® Red. Subsequently cells were fixed to the coverslips using 4% paraformaldehyde and permeabilized with Triton X-100. Finally the nucleus was stained with DAPI (0.2 mg/mL) for 5 minutes in the dark and the cells visualized.

3.2.12. Statistical Analysis

At least three batches of each polyplexes and PM were produced and characterized and the results expressed as the mean±standard deviation (SD). For biological studies, at least 3 replicates, each involving at least two technical replicates were involved in final results expressed as the mean±SD. Statistical analysis was performed in Microsoft Office Excel™ 2007 using the unpaired Student's t-test. Differences were regarded as statistically significant when p-value were smaller than 0.05.

3.3. Results

3.3.1. CS-based Polyplexes

For a preliminary screening of the best conditions and type of CS to form polyplexes for siRNA delivery, the association efficiency, the *in vitro* cytotoxicity, and the biological efficacy of the different polyplexes was determined.

As possible to observe in the **Figure 3.3**, all the CS were able to efficiently complex the siRNA. A significant decrease in the concentration of free siRNA is observed spectrophotometrically upon complexation with CS at low ratios. Additionally, in the agarose gel assay, a small amount of free siRNA is only detected when 1:1 ratio of CS:siRNA is used. Since the low N/P ratios that are sufficient to complex the siRNA are not enough to obtain biological efficacy, higher N/P ratios were tested in terms of cell toxicity in MDA-MB-231 breast cancer cells**. Figure 3.4** demonstrate that CL113, G113, CL213 and G213 have a similar toxicity pattern, being the N/P ratio 80 the maximum N/P ratio that can be used without cause severe toxicity. The Glycol-CS appears to be the one causing the higher toxicity even at lower N/P ratios.

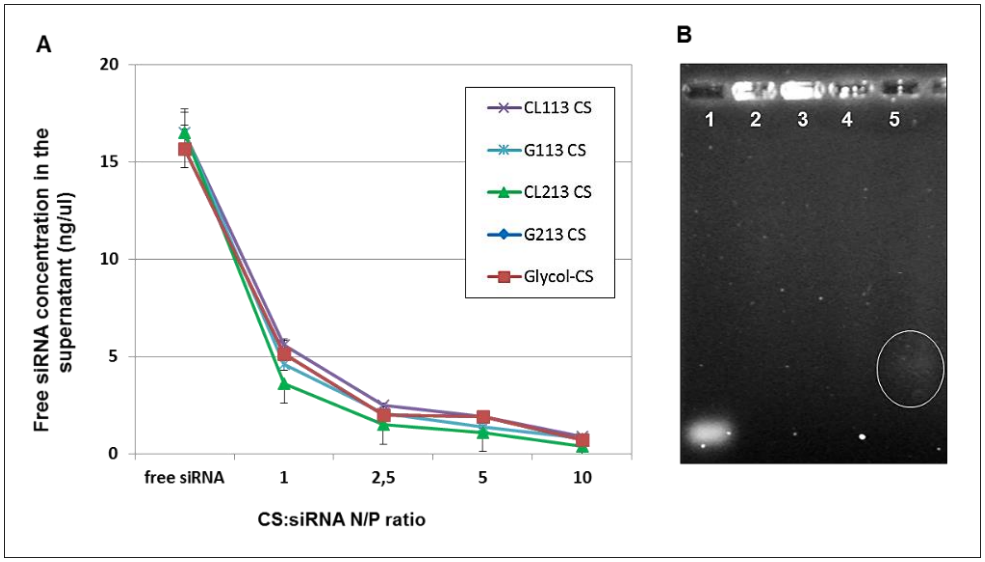


Figure 3. 3. Polyplexes association efficiency. A) Concentration of free siRNA detected in the supernatant by spectrophotometry after formulation filtration by centrifugation using different N/P ratios. Results are expressed as mean±SD, n=3. B) Agarose gel electrophoresis for the CL213-based polyplex. 1 – free siRNA; 2 – 1:10 ratio; 3 – 1:5 ratio; 4 – 1:2.5 ratio; 5 – 1:1 ratio. In number 5, it is possible to see some free siRNA (marked with the circle).

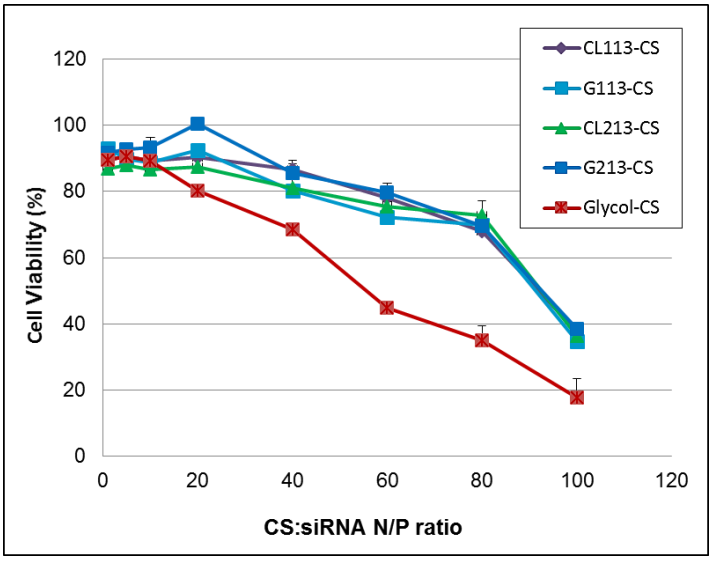


Figure 3. 4. *In vitro* cytotoxicity of CS-siRNA polyplexes at different N/P ration in MDA-MB-231 cells. Results are expressed as mean±SD, n=3.

As described in **Table 3.2**, the most promising polyplexes, i.e. the ones that were able to cause a visible decrease in the number of cells expressing GFP without promote significant toxicity, were obtained with the following conditions: GFP-siRNA at a concentration of 200 nM in the well, complexed with CL213 and G213 CS at a N/P ratio of 80 and pH 4.5, and at a post-transfection incubation time of 72 hours.

Table 3. 2. Summary of the different tested conditions regarding the CS-based polyplexes.

Tested Conditions		Observations
siRNA final conc. in the well	50 nM	No effect
	100 nM	No effect
	200 nM	Effective (at certain conditions)
Polyplexes	Glycol-CS	Toxic (at ratios > 60)
	G113	No effect
	CL113	No effect
	G213	Effective (at certain conditions)
	CL213	Effective (at certain conditions)
N/P ratios	< 10	No effect
	20	No effect
	30	No effect
	40	No effect
	60	No effect
	80	Knockdown detected (at certain toxicity)
	100	Toxicity
	>100	Highly toxic
pH	4.5 (in acetate buffer)	Higher efficacy
	7 (in water)	Low effect
Time-points	24 hours	No effect
	48 hours	No effect
	72 hours	Knockdown detected
Micelles	CL213 (N/P 80, pH 4.5) + F127 (1%) Time-point 72 h	Effect < than Lipofectamine® 2000

In **Figure 3.5** are depicted the results of the silencing efficacy of the CL213-based GFP-siRNA polyplexes formed at the chosen optimal conditions. It is observed a partial silencing in the expression of GFP 72 hours after transfection with the polyplexes. On the contrary, no decrease in GFP expression was observed in the cells transfected with the siC. It is also possible to observe that for the cells transfected with the Lipofectamine® 2000 the GFP silencing is much stronger than the one obtained with the polyplexes. Similar results were obtained with the polyplexes composed by G213; however the silencing effect was not so reproducible. On the other hand, no significant silencing efficacy was observed with the other types of CS tested. The MW of CS seems to be involved in its transfection efficiency, since higher transfections efficiencies were obtained with the CS presenting the higher MW, possibly due to the higher capacity to complex and protect the genetic material.

After choosing the best conditions, for polyplexes preparation, in terms of cytotoxicity and biological efficacy, they were further characterized in terms of their physicochemical features.

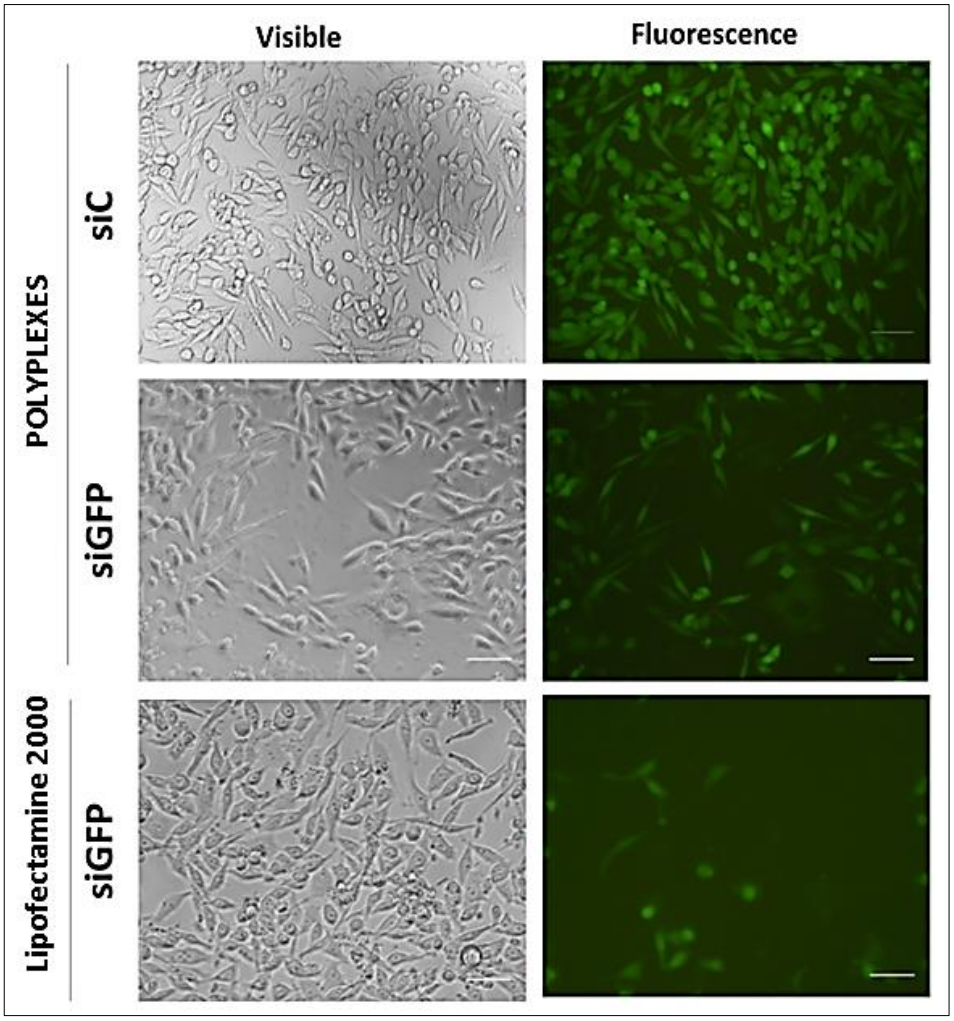


Figure 3. 5. Polyplexes CS-siRNA silencing efficacy 72 hours after transfection in RXO-C colon cancer cells expressing GFP. The biological activity was assessed based on the reduction of GFP expression in the cells transfected with CS polyplexes with GFP-siRNA versus siC, using a fluorescence microscope. Lipofectamine® 2000-based complexes were used as the positive control.

Parameters	Polyplexes CS-siRNA
Md(nm)	16.6 ± 0.8
Pdi	0.19 ± 0.07
Zp (mV)	+15.2 ± 1.7

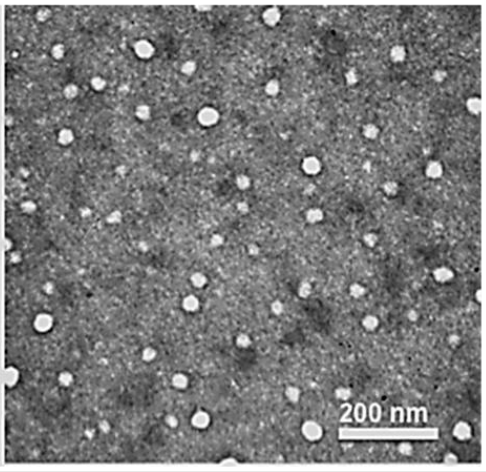


Figure 3. 6. Polyplexes physicochemical characterization using CL213 CS. Mean diameter (md), polydispersity index (Pdi) and Zeta potential (Zp) values for polyplexes Cs-siRNA (results are expressed as mean±SD, n=3) and TEM image of the same formulation.

The polyplexes of CS-siRNA produced with the CL213-CS shown to be small (< 17 nm) and positively charged (+ 15 mV) particles with spherical shape (**Figure 3.6**). The next step of the work consisted in the conjugation of this polyplexes with Pluronic® based micelles in order to improve the siRNA protection and biological activity.

3.3.2. Pluronic®-based Micelles Containing CS-polyplexes

Pluronic®-based empty micelles were prepared using different polymers. The micelles presented a size lower than 150 nm (inferior to 50 nm for Pluronic® F108 and F127) and near neutral surface charge (**Table 3.3**).

Table 3. 3. Physicochemical characterization of different Pluronic®-based micelles. Results are expressed as mean±SD, n=3.

Polymer	Md (nm)	Pdi	Zp (mV)
F68	128.6±31.2	0.5±0.2	-2.9±1.0
F108	42.6±8.4	0.3±0.2	-2.1±1.2
F127	25.0±3.4	0.1±0.05	-2.7±1.8

Due to the high polydispersity of micelles and a low reproducibility obtained with the F68 polymer, this polymer was discarded from further studies.

Concerning the cytotoxicity assessment, both Pluronic® F108 and F127 presented an IC_{50} superior to 10 mg/ml **(Figure 3.7)**, but since Pluronic® F127 originates the micelles presenting the lowest values of md and with the less polydispersed population, this polymer was the chosen one for the production of micelles combined with the CS-based polyplexes.

For further experiments CL213-CS-based GFP-siRNA polyplexes were formed at the chosen optimal conditions previously referred. These polyplexes were used to hydrate the Pluronic® F127 film, forming the final micelles-based formulation (CS-siRNA-Pluronic®).

For the biological studies, these micelles were posteriorly incubated with RXO-C colon cancer cells expressing GFP, being possible to observe that GFP expression was silenced, conversely with what happens in cells transfected with the siC (**Figure 3.8**). Despite the improvement in the silencing efficacy observed in the presence of Pluronic®, the efficacy of the systems is still lower that the previously obtained using the positive control (Lipofectamine® 2000).

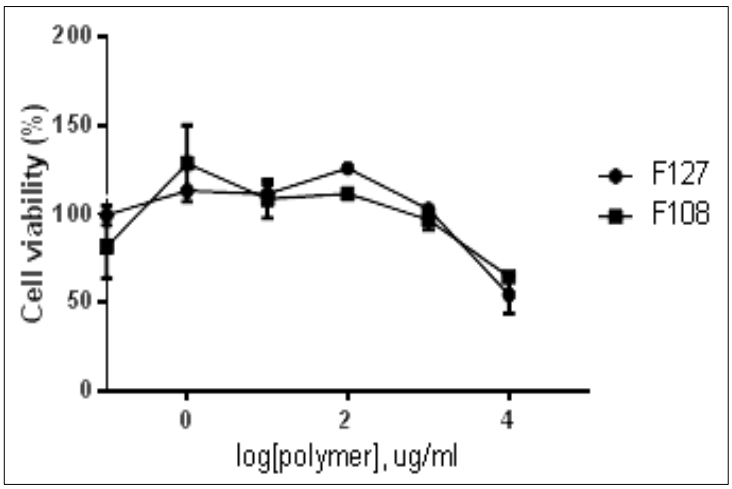


Figure 3. 7. Comparative cytotoxicity and IC_{50} values of Pluronic® F127 and F108 in MDA-MB-231 cells. Results are expressed as mean±SD, n=3. F127 and F108 IC_{50} > 10 mg/ml.

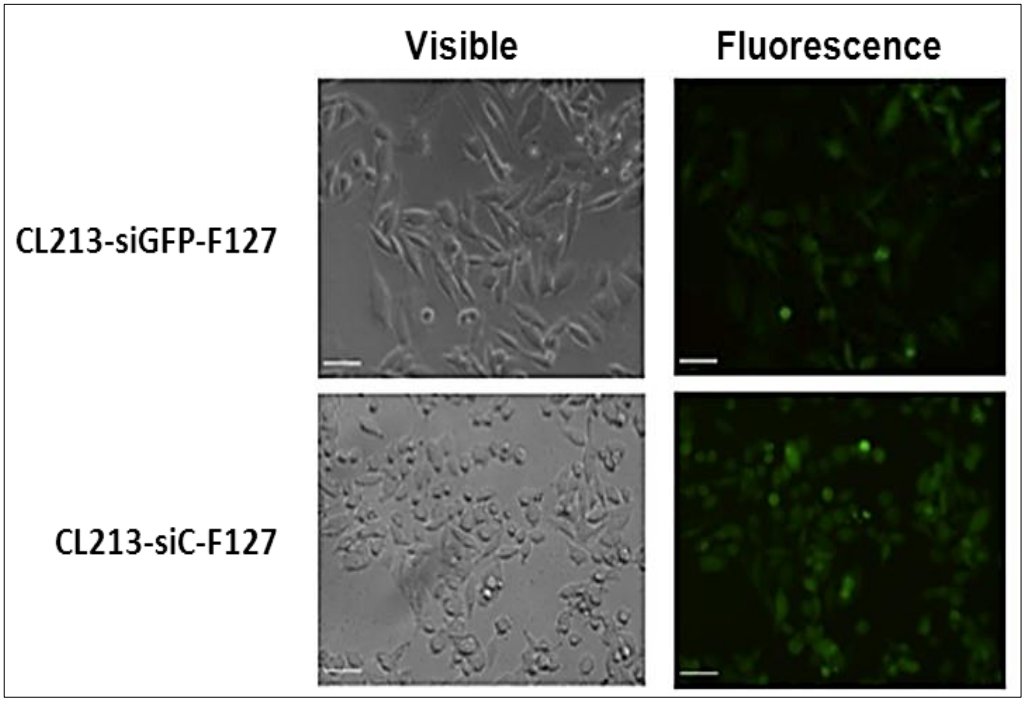


Figure 3. 8. CS-siRNA-Pluronic® micelles silencing efficacy 72 hours after transfection with the siGFP and siC in RXO-C colon cancer cells.

3.3.3. PEI-based Systems

Besides the micelles prepared by the FH technique described in the **Chapter 5**, it was decided to produce and characterize micelles prepared also by the direct dissolution method in order to assess the possibility of producing the formulations without using organic solvents. For the design of these nanoparticles, the different conditions tested and results obtained are summarized in **Table 3.4**.

Table 3. 4. Summary of the different tested conditions regarding the branched PEI-based systems.

Tested Conditions		Observations
siRNA final conc. in the well	200 nM	Effective (at certain conditions)
Polymers combinations	PEI 10K	Low effect
	PEI 25K	Low effect
	PEI 10K + F127 (0.5%)	Effect < than Lipofectamine® 2000
	PEI 25 K + F127 (0.5%)	Effect < than Lipofectamine® 2000
	PEI 10K + F127 (1%)	Effective (at N/P 50)
	PEI 25 K + F127 (1%)	Effective (at N/P 25 and 50)
N/P ratios PEI:siRNA	5	No effect
	25	Effective only for PEI 25K
	50	Effective
	75	Toxic

In the assessment of the biological efficacy no significant differences were observed between both types of PEI **(Figure 3.9)**, but since the 10K branched PEI presented lower toxicity profile (30, 31), it was chosen for further studies.

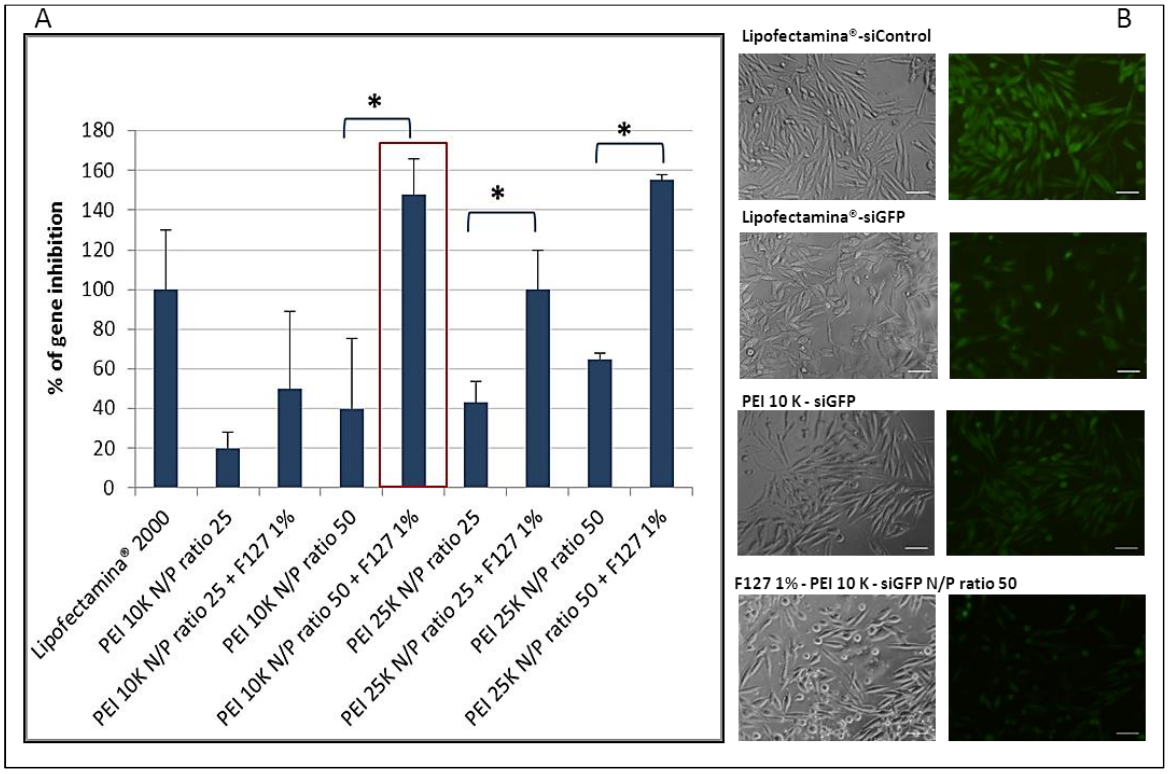


Figure 3. 9. PEI-siRNA polyplexes and PEI-siRNA-Pluronic® micelles (obtained by DM) silencing efficacy in GFP expressing RXO-C cells. A) Differences on the intensity of gene expression upon incubation with different formulations using as reference the Lipofectamine® 2000. B) Fluorescent microscopy photos confirming the GFP silencing efficacy of the selected formulation. No gene inhibition was observed in cells transfected with siC. The values of GFP silencing were normalized to the ones obtained with Lipofectamine® 2000. The red square represents the selected formulation. Results are expressed as mean±SD (n≥3). * p≤0.05 compared to the polyplexes without Pluronic® F127.

Formulations composed by PEI:siRNA polyplexes of N/P ratio 50 present higher transfection efficacy then polyplexes at lower N/P ratios **(Figure 3.9)**. Also, the presence of 1% Pluronic® F127 in the form of micelles (prepared by DM) improved significantly the transfection efficiency of the polyplexes as observed both quantitatively and qualitatively by an increase of the gene expression inhibition **(Figure 3.9)**. Thus, Pluronic® 127 micelles encapsulating PEI:siRNA polyplexes at a N/P ratio 50 were chosen to proceed with further studies and posteriorly characterized in terms of its internalization profile, md, Pdi, zeta potential, serum stability, and entrapment efficiency.

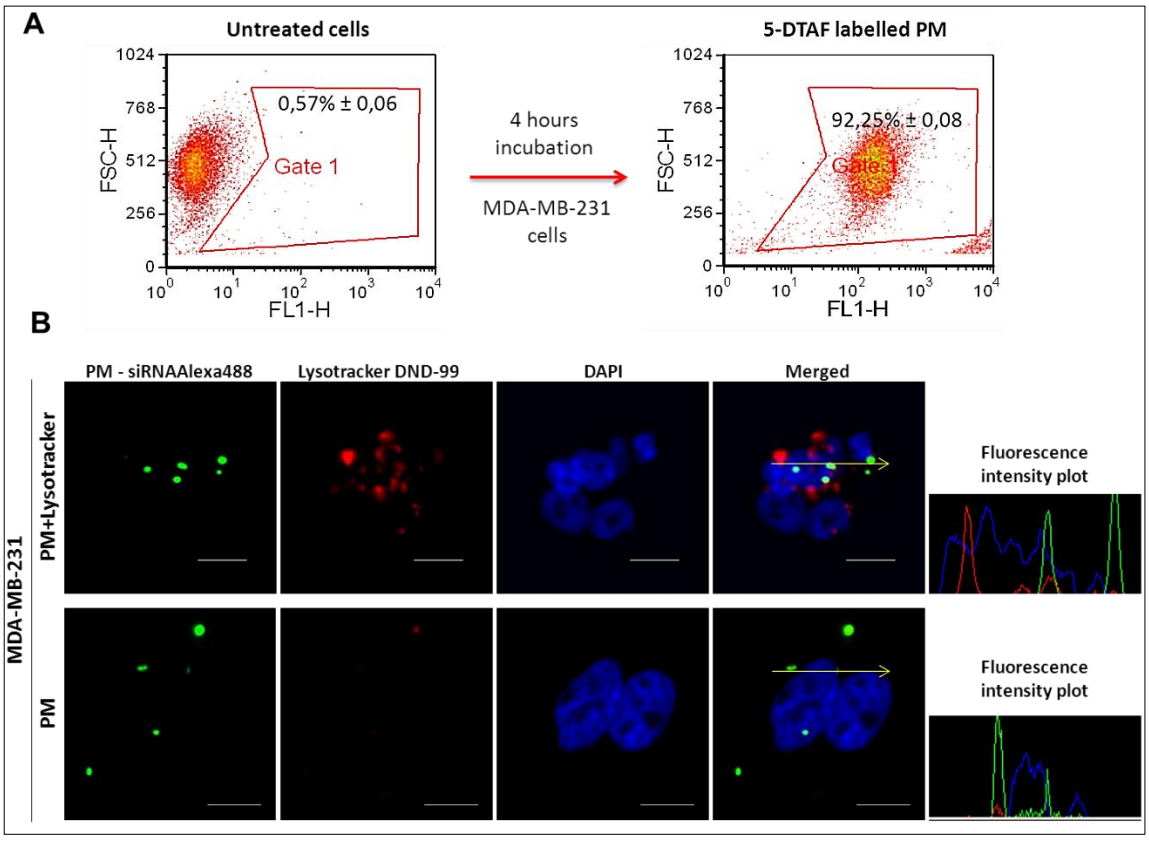


Figure 3. 10. PEI-siRNA-Pluronic® internalization behavior. A) Flow cytometry analysis of labelled particles internalization. B) Confocal Microscopy analysis of particles internalization containing a fluorescent siRNA (siRNA-AlexaFluor 488). The yellow arrow illustrates the respective intracellular distribution of nanoparticle and Lysotracker (DND-99, red).

The internalization of the PEI-siRNA-Pluronic® micelles was assessed quantitatively by flow cytometry **(Figure 3.10A)** and qualitatively by confocal microscopy **(Figure 3.10B)**. For the FACS experiments, cells were incubated with 5-DTAF-labeled micelles during 4 hours. For the qualitative analysis, MDA-MB-231 cells were incubated during 4 hours with micelles carrying a fluorescent-labeled siRNA (siRNA-AlexaFluor 488) and observed by confocal microscopy. As expected, in both assays it is possible to observe total micelles internalization after the incubation time. In the confocal microscopy images it is also

possible to see that the green-labelled siRNA is not co-localized with the endosomal vesicles, being already available in the cytoplasm where will exert its effect.

Formulation	Md (nm)	Pdi	ZP (mV)	AE (%)
PM-siRNA	60,28 ± 8,65	0,51 ± 0,059	-0,21 ± 0,060	93,02 ± 0,12
PM	73,22 ± 9,54	0,28 ± 0,078	3,81 ± 0,96	—

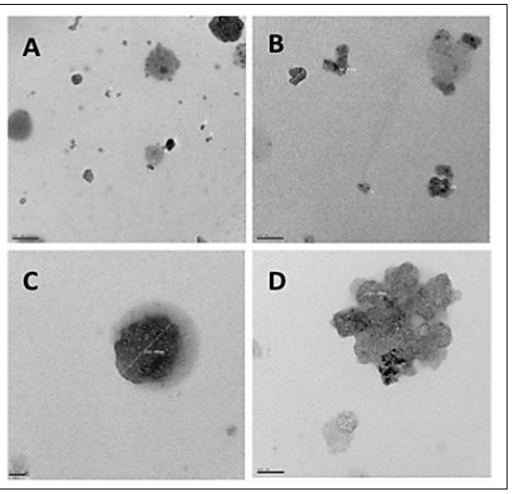


Figure 3. 11. Physicochemical characterization of PEI-siRNA-Pluronic® micelles. The table shows the values of mean diameter, polydispersity index and zeta potential for the loaded and the unloaded formulation and the value of association efficiency calculated based on the undirected method described before. In the TEM images represent: A) Different particles subpopulations, B) Particles with around 70 nm, C) Particles with around 200 nm, D) Aggregates. Results are expressed as mean±SD, n=3.

Regarding the physicochemical characterization of the chosen formulation, it is possible to see in **Figure 3.11** that these nanoparticles present a higher size and Pdi compared to the micelles prepared by FH technique **(Figure 5.2** of **Chapter 5)**. These results were confirmed by TEM analysis that shows different sub-populations of particles with different sizes and also the presence of aggregates. Despite the high tendency to aggregate, that result in their low storage stability, these nanoparticles present high siRNA entrapment efficiency **Figure 3.11**.

The results of stability of particles in serum are depicted in **Figure 3.12**. It can be noted an increase in the mean diameter of particles over time after the first 6 hours of incubation, which indicates the formation of aggregates with the serum proteins.

Despite the higher size and Pdi compared to the micelles prepared by FH technique, micelles prepared by DM have shown to be also less stable and more toxic (**Table 3.5**).

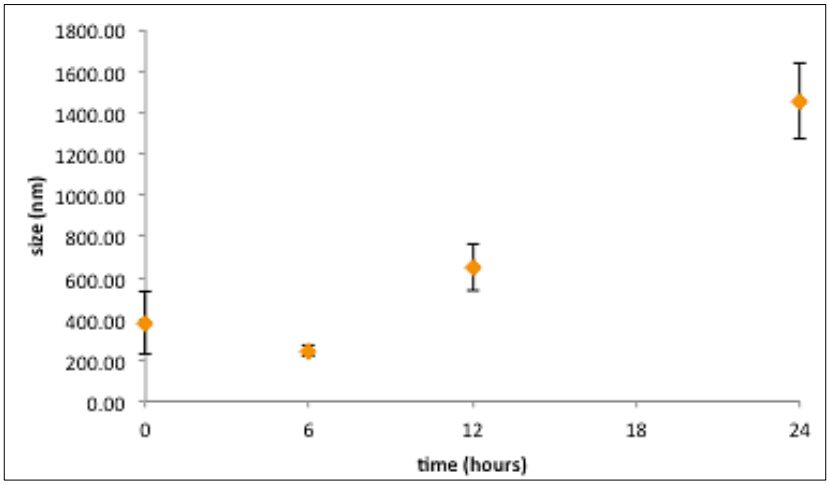


Figure 3. 12. Serum stability of PEI-siRNA-Pluronic® formulation. The graphic despites the mean diameter of micelles after incubation with serum at different time-points. Results are expressed as mean±SD, n=3.

Table 3. 5. Main differences between micelles obtained through preparation methods. DM: direct method; FH: thin-film hydration technique.

	DM	FH
Preparation Method	Simple	Simple (more steps)
Mean Diameter/Zeta	<100 nm	<100 nm
Polydispersity	> 0.3	< 0.2
Encapsulation Efficiency	> 95%	> 95%
Internalization	> 95%, 6 hours	> 95%, 6 hours
Biological Efficacy	≥ Lipofectamine® 2000	≥ Lipofectamine® 2000
Stability	< 1 day	> 1 week
Toxicity	↑↑↑	-
Reproducibility	No	Yes

3.4. Discussion

The objective of the present Chapter relies on the development of an efficient and safe siRNA delivery system. As well-known, a good gene delivery system requires the presence of a cationic component able to complex and condense the genetic material in order to promote its cellular internalization and exert its biological effect (32-34).

Different polymeric-based formulations were designed and tested over the period of work to reach the one that best fits the proper characteristics for siRNA delivery. Although several formulations were dropped during the time course of the work due to their toxicity and/or low biological efficacy, all of them supplied important information that allowed us to reach the system with which we are currently working, and whose results are described in **Chapter 5**. The **Figure 3.13** summarizes different strategies and the related challenges that we have

faced during the formulation process. Nevertheless, some of the dropped formulations, if improved and properly optimized in the future, have the potential to become interesting gene delivery systems.

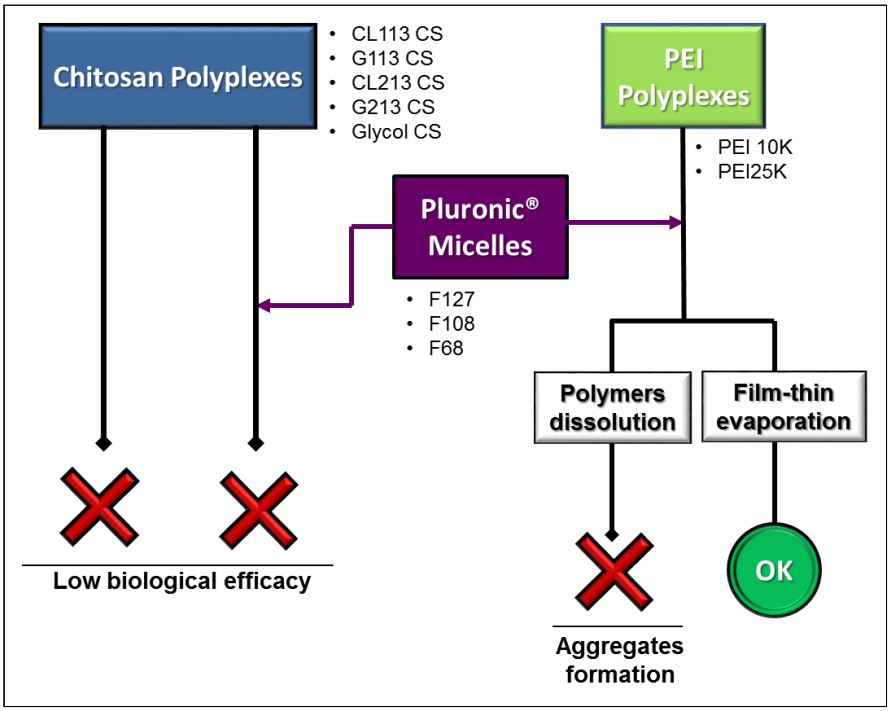


Figure 3. 13. Diagram resuming the different developed tested formulations for gene therapy and the main results.

Since CS is a well-established biopolymer with recognized advantages in terms of gene delivery, our first approach consisted in the production of polyplexes composed by CS and siRNA (CS-siRNA) (35, 36). Different types of CS were tested at different conditions of concentration, pH and N/P ratio, in order to achieve the correct silencing efficacy/toxicity balance when compared with the positive control of transfection (Lipofectamine® 2000). RXO-C colon cancer cells expressing GFP were used to test the silencing efficacy of polyplexes prepared with a GFP-siRNA. The biological activity was assessed based on the reduction of GFP expression in the transfected cells with GFP-siRNA versus PM-siC, using a fluorescence microscope. The ones presenting the best characteristics were the CS with higher MW, which could be explained by its higher positive charge and capacity to better complex and protect the siRNA. Similar results were observed by others using plasmids and siRNA (37-41). However, the biological efficacy of the CS polyplexes observed was low. Although the higher MW CS seems to condense and protect the siRNA to be delivered to cells, it is possible that the strong interaction results in a low dissociation between the genetic material and CS after endosomal disruption, and consequentely low biological efficacy. As referred previously and observed by others, it is necessary a balance between

the MW and DD able to efficiently complex and protect the genetic material, promote the endosomal escape, and release the genes into the cytoplasm (40, 41). Regarding the polyplexes association efficiency, different polymer:siRNA ratios were tested, being possible to confirm the so well recognized capacity of CS to complex OGN, even at lower ratios (38, 39). Although a 1:2.5 ratio is enough to complex the siRNA, the GFP reporter silencing assays demonstrate that in order to promote the endosomal swelling and to obtain biological efficacy it is necessary to use much higher polymer concentrations (N/P ratio 80). This highlights that although the complexation of the genetic material is essential for its proper delivery, is not the unique condition for the existence of biological effect.

The final goal of the work was to find the most effective polyplex to be posteriorly combined with another nanoparticulate system, namely PM composed of Pluronic®. These types of polymers have been described as enhancers of the transfection of genetic material; however, since poloxamers present neutral charge at the physiological pH, as estimated in silico using the Marvin Suite software (ChemAxon, Hungary), it was necessary to introduce a cationic component to formulation in order to garnish it with the required positive charge to complex the anionic siRNA. Thus, the developed CS- and PEI-based polyplexes were combined with Pluronic®-based micelles previously developed by members of the group for delivery of biopharmaceuticals (42).

Different Pluronic® (F68, F108, F127) were tested, being the Pluronic® F127 selected due to its better technological features. The smaller size and Pdi of F127-based micelles can be explained by its lower CMC, hydrophilic-lipophilic balance (HLB) and CMT (24 ºC for 1% w/v (37, 43)), whereas F68 presents the higher CMC and CMT (54-56.21 ºC for 1% w/v (44)) and consequently higher diameter. The same behavior was observed previously (45). Although, the silencing efficiency of the CS-based polyplexes appears to be improved with the presence of Pluronic® F127 in the formulation, it is lower then the obtained with Lipofectamine® 2000. Taking into account that the presence of Pluronic® improved the transfection efficiency of CS-based polyplexes, the next step of the present work consisted in the substitution of CS for another cationic polymer, in this case PEI.

In order to rapidly screen different formulation compositions regarding its transfection efficiency, the reduction of fluorescence in RXO-C colon cancer cells GFP expressing cells after transfection was quantified using a fluorescent microplate reader and qualitatively assessed on a fluorescent microscope. It was observed that PEI polyplexes were able to inhibit a certain level of gene expression, presenting the N/P ratio 50 higher transfection efficiency than N/P ratio 25. Also, the presence of 1% Pluronic® (w/v) in the form of micelles improved significantly the transfection efficiency of the polyplexes as observed both quantitatively and qualitatively by an increase of the GFP gene expression inhibition. The

formulations composed by PEI:siRNA polyplexes of N/P ratio 50 with F127 1% (w/v) present similar transfection efficacy and GFP silencing than Lipofectamine® 2000.

Since no significant differences in terms of biological efficacy were observed between both types of PEI, the 10K branched PEI was chosen due to its recognized lower toxicity profile (30, 31). Thus, after the first approach based on the assessment of transfection efficiency, the formulation where PEI 10k was complexed with the siRNA at the N/P ratio of 50 and further incorporated in Pluronic® F127-based PM was selected to further technological and biological experiments.

Contrary to the complex chemical modifications proposed by other studies (46, 47), in this work we propose a simple association of PEI-based polyplexes with Pluronic® F127 based PM, that significantly increased the polyplexes biological efficacy. This result can be explained by the reported capacity of poloxamers to enhance themselves the transfection efficiency of genetic material. Although not completely understood and explained, poloxamers present the capacity to interfere to some extent to the cell membranes reducing its structure and microviscosity/fluidity (33, 48), and to improve the escape from the endosomal compartment via membrane disruption and pore formation (49, 50). Poloxamers with an intermediate HLB value like F127 (HLB=18-23), have shown higher ability to interfere with membranes than poloxamers with higher HLB like F68 and F108 (HLB>24) (51).

In this work, PEI-containing micelles were prepared by two methods in order to compare their characteristics and to choose the best preparation method: DM (direct dissolution method described in this chapter) and FH (thin-film hydration technique described in **Chapter 5**. Micelles prepared by DM present the advantage of being prepared by a simpler method avoiding also the use of organic solvents; however, they have shown higher size and polydispersity, as well as lower stability and higher toxicity profile when compared to the micelles presented in **Chapter 5**, which could be related to a less effective coating of PEI-polyplexes.

3.5. Conclusions

This chapter described the rational design and experimental sequence beyond the development of a nanocarrier system for siRNA intracellular delivery. Different cationic polymers for the production of polyplexes and different systems to carry the polyplexes were tested.

The choice of PM composed by Pluronic® as a possible delivery system is based on their amphiphilic properties opening the possibility to design a multifunctional-targeted particle

that can be used in the future for multi-therapy, i.e. the blend of gene and chemotherapy in one single nanoparticle formulation.

It results clear that the presence of Pluronic® in the formulations improved the biological efficacy of CS-based polyplexes. Nevertheless, even this combination presented limited transfection efficiency. Thus, the well-establish PEI was used for the production of polyplexes and for the development of PEI-siRNA-Pluronic® systems.

In the present work was possible to develop a formulation composed by PEI 10k branched-based polyplexes at an N/P ratio of 50 combined with micelles of Pluronic® F127 to a final concentration of 1% (w/v) presenting adequate technological properties, biological efficacy and safety profile. It was also possible to emphasize the importance of the presence of Pluronic® F127 in the formulation not only to improve the silencing activity but also to reduce the PEI-associated toxicity.

The direct dissolution method to prepare micelles is easiest and less time consuming, in addition of avoiding organic solvents; however it presented some disadvantages when compared with the FH technique to produce the PEI-siRNA-Pluronic® systems**.** Despite the observed gene silencing efficacy and safety profile, the higher size and polydispersity of particles associated to high physical instability observed make us decide to change the method of micelles production to FH, maintaining the composition of the formulations **(Chapter 5)**.

3.6. References

1. Mao S, Sun W, Kissel T. Chitosan-based formulations for delivery of DNA and siRNA. Adv Drug Deliv Rev. 2010;62(1):12-27.

2. Rudzinski WE, Aminabhavi TM. Chitosan as a carrier for targeted delivery of small interfering RNA. Int J Pharm. 2010;399(1-2):1-11.

3. Holzerny P, Ajdini B, Heusermann W, Bruno K, Schuleit M, Meinel L, et al. Biophysical properties of chitosan/siRNA polyplexes: profiling the polymer/siRNA interactions and bioactivity. J Control Release. 2012;157(2):297-304.

4. Park IK, Park YH, Shin BA, Choi ES, Kim YR, Akaike T, et al. Galactosylated chitosan-graft-dextran as hepatocyte-targeting DNA carrier. J Control Release. 2000;69(1):97-108.

5. Kim TH, Ihm JE, Choi YJ, Nah JW, Cho CS. Efficient gene delivery by urocanic acid-modified chitosan. J Control Release. 2003;93(3):389-402.

6. Kim TH, Kim SI, Akaike T, Cho CS. Synergistic effect of poly(ethylenimine) on the transfection efficiency of galactosylated chitosan/DNA complexes. J Control Release. 2005;105(3):354-66.

7. Wong K, Sun G, Zhang X, Dai H, Liu Y, He C, et al. PEI-g-chitosan, a novel gene delivery system with transfection efficiency comparable to polyethylenimine in vitro and after liver administration in vivo. Bioconjug Chem. 2006;17(1):152-8.

8. Lin A, Liu Y, Huang Y, Sun J, Wu Z, Zhang X, et al. Glycyrrhizin surface-modified chitosan nanoparticles for hepatocyte-targeted delivery. Int J Pharm. 2008;359(1-2):247-53.

9. Gabizon A, Shmeeda H, Horowitz AT, Zalipsky S. Tumor cell targeting of liposome-entrapped drugs with phospholipid-anchored folic acid-PEG conjugates. Adv Drug Deliv Rev. 2004;56(8):1177-92.

10. Moreira C, Oliveira H, Pires LR, Simões S, Barbosa MA, Pêgo AP. Improving chitosan-mediated gene transfer by the introduction of intracellular buffering moieties into the chitosan backbone. Acta Biomater. 2009;5(8):2995-3006.

11. Akinc A, Thomas M, Klibanov AM, Langer R. Exploring polyethylenimine-mediated DNA transfection and the proton sponge hypothesis. J Gene Med. 2005;7(5):657-63.

12. Roesler S, Koch FP, Schmehl T, Weissmann N, Seeger W, Gessler T, et al. Amphiphilic, low molecular weight poly(ethylene imine) derivatives with enhanced stability for efficient pulmonary gene delivery. J Gene Med. 2011;13(2):123-33.

13. Martin I, Dohmen C, Mas-Moruno C, Troiber C, Kos P, Schaffert D, et al. Solid-phase-assisted synthesis of targeting peptide-PEG-oligo(ethane amino)amides for receptor-mediated gene delivery. Org Biomol Chem. 2012;10(16):3258-68.

14. Godbey WT, Wu KK, Mikos AG. Size matters: molecular weight affects the efficiency of poly(ethylenimine) as a gene delivery vehicle. J Biomed Mater Res. 1999;45(3):268-75.

15. Thomas M, Klibanov AM. Enhancing polyethylenimine's delivery of plasmid DNA into mammalian cells. Proc Natl Acad Sci U S A. 2002;99(23):14640-5.

16. Mao S, Neu M, Germershaus O, Merkel O, Sitterberg J, Bakowsky U, et al. Influence of polyethylene glycol chain length on the physicochemical and biological properties of poly(ethylene imine)-graft-poly(ethylene glycol) block copolymer/SiRNA polyplexes. Bioconjug Chem. 2006;17(5):1209-18.

17. Dehshahri A, Oskuee RK, Shier WT, Hatefi A, Ramezani M. Gene transfer efficiency of high primary amine content, hydrophobic, alkyl-oligoamine derivatives of polyethylenimine. Biomaterials. 2009;30(25):4187-94.

18. Grayson AC, Doody AM, Putnam D. Biophysical and structural characterization of polyethylenimine-mediated siRNA delivery in vitro. Pharm Res. 2006;23(8):1868-76.

19. Oskuee RK, Philipp A, Dehshahri A, Wagner E, Ramezani M. The impact of carboxyalkylation of branched polyethylenimine on effectiveness in small interfering RNA delivery. J Gene Med. 2010;12(9):729-38.
20. Malek A, Czubayko F, Aigner A. PEG grafting of polyethylenimine (PEI) exerts different effects on DNA transfection and siRNA-induced gene targeting efficacy. Journal of Drug Targeting. 2008;16(2):124-39.
21. Moghimi SM, Hunter AC. Poloxamers and poloxamines in nanoparticle engineering and experimental medicine. Trends Biotechnol. 2000;18(10):412-20.
22. Kabanov AV, Batrakova EV, Alakhov VY. Pluronic block copolymers as novel polymer therapeutics for drug and gene delivery. J Control Release. 2002;82(2-3):189-212.
23. Kabanov A, Zhu J, Alakhov V. Pluronic block copolymers for gene delivery. Advances in genetics. 2005;53:231-61.
24. Batrakova EV, Kabanov AV. Pluronic block copolymers: evolution of drug delivery concept from inert nanocarriers to biological response modifiers. J Control Release. 2008;130(2):98-106.
25. Chen YC, Jiang LP, Liu NX, Ding L, Liu XL, Wang ZH, et al. Enhanced gene transduction into skeletal muscle of mice in vivo with pluronic block copolymers and ultrasound exposure. Cell Biochem Biophys. 2011;60(3):267-73.
26. Wang F, Li K, Chen Y. Gene transfection mediated by ultrasound and Pluronic P85 in HepG2 cells. J Huazhong Univ Sci Technolog Med Sci. 2007;27(6):700-2.
27. Yamagata M, Kawano T, Shiba K, Mori T, Katayama Y, Niidome T. Structural advantage of dendritic poly(L-lysine) for gene delivery into cells. Bioorg Med Chem. 2007;15(1):526-32.
28. Inoue Y, Kurihara R, Tsuchida A, Hasegawa M, Nagashima T, Mori T, et al. Efficient delivery of siRNA using dendritic poly(L-lysine) for loss-of-function analysis. J Control Release. 2008;126(1):59-66.
29. Andrade F, Neves J, Gener P, Schwartz S, Jr., Ferreira D, Oliva M, et al. Biological assessment of self-assembled polymeric micelles for pulmonary administration of insulin. Nanomedicine : nanotechnology, biology, and medicine. 2015;11(7):1621-31.
30. Paul A, Eun C-J, Song JM. Cytotoxicity mechanism of non-viral carriers polyethylenimine and poly-l-lysine using real time high-content cellular assay. Polymer. 2014;55(20):5178–88.
31. Symonds P, Murray JC, Hunter AC, Debska G, Szewczyk A, Moghimi SM. Low and high molecular weight poly(L-lysine)s/poly(L-lysine)-DNA complexes initiate mitochondrial-mediated apoptosis differently. FEBS Lett. 2005;579(27):6191-8.

32. Tros de Ilarduya C, Sun Y, Düzgüneş N. Gene delivery by lipoplexes and polyplexes. Eur J Pharm Sci. 2010;40(3):159-70.

33. Iyer AK, Duan Z, Amiji MM. Nanodelivery systems for nucleic acid therapeutics in drug resistant tumors. Molecular pharmaceutics. 2014;11(8):2511-26.

34. Rafael DF, Andrade F, Arranja A, Luís AS, Videira M. Lipoplexes and Polyplexes: Gene Delivery Applications. . Encyclopedia Biomedical Polymers and Polymeric Biomaterials, Taylor and Francis Group 2014.

35. Huang M, Fong CW, Khor E, Lim LY. Transfection efficiency of chitosan vectors: effect of polymer molecular weight and degree of deacetylation. J Control Release. 2005;106(3):391-406.

36. Lavertu M, Méthot S, Tran-Khanh N, Buschmann MD. High efficiency gene transfer using chitosan/DNA nanoparticles with specific combinations of molecular weight and degree of deacetylation. Biomaterials. 2006;27(27):4815-24.

37. Andrade F, Fonte P, Oliva M, Videira M, Ferreira D, Sarmento B. Solid state formulations composed by amphiphilic polymers for delivery of proteins: characterization and stability. Int J Pharm. 2015;486(1-2):195-206.

38. Mao S, Sun W, Kissel T. Chitosan-based formulations for delivery of DNA and siRNA. Adv Drug Deliv Rev. 2010;62(1):12-27.

39. Rudzinski WE, Aminabhavi TM. Chitosan as a carrier for targeted delivery of small interfering RNA. International journal of pharmaceutics. 2010;399(1-2):1-11.

40. Köping-Höggård M, Tubulekas I, Guan H, Edwards K, Nilsson M, Vårum KM, et al. Chitosan as a nonviral gene delivery system. Structure-property relationships and characteristics compared with polyethylenimine in vitro and after lung administration in vivo. Gene Ther. 2001;8(14):1108-21.

41. Köping-Höggård M, Vårum KM, Issa M, Danielsen S, Christensen BE, Stokke BT, et al. Improved chitosan-mediated gene delivery based on easily dissociated chitosan polyplexes of highly defined chitosan oligomers. Gene Ther. 2004;11(19):1441-52.

42. Katas H, Alpar HO. Development and characterisation of chitosan nanoparticles for siRNA delivery. J Control Release. 2006;115(2):216-25.

43. Andrade F, das Neves J, Gener P, Schwartz S, Ferreira D, Oliva M, et al. Biological assessment of self-assembled polymeric micelles for pulmonary administration of insulin. Nanomedicine. 2015;11(7):1621-31.

44. Lin Y, Alexandridis P. Temperature-dependent adsorption of Pluronic F127 block copolymers onto carbon black particles dispersed in aqueous media. J Phys Chem B. 2002;106:10834-44.

45. Tsui H-W, Wang J-H, Hsu Y-H, Chen L-J. Study of heat of micellization and phase separation for Pluronic aqueous solutions by using a high sensitivity differential scanning calorimetry. Colloid and Polymer Science. 2010;288:1687-96.

46. Hu Y, Zhou D, Li C, Zhou H, Chen J, Zhang Z, et al. Gene delivery of PEI incorporating with functional block copolymer via non-covalent assembly strategy. Acta biomaterialia. 2013;9(2):5003-12.

47. Wen Y, Pan S, Luo X, Zhang W, Shen Y, Feng M. PEG- and PDMAEG-graft-modified branched PEI as novel gene vector: synthesis, characterization and gene transfection. Journal of biomaterials science Polymer edition. 2010;21(8-9):1103-26.

48. Williford JM, Wu J, Ren Y, Archang MM, Leong KW, Mao HQ. Recent advances in nanoparticle-mediated siRNA delivery. Annual review of biomedical engineering. 2014;16:347-70.

49. Demina T, Grozdova I, Krylova O, Zhirnov A, Istratov V, Frey H, et al. Relationship between the structure of amphiphilic copolymers and their ability to disturb lipid bilayers. Biochemistry. 2005;44(10):4042-54.

50. Batrakova EV, Li S, Alakhov VY, Miller DW, Kabanov AV. Optimal structure requirements for pluronic block copolymers in modifying P-glycoprotein drug efflux transporter activity in bovine brain microvessel endothelial cells. The Journal of pharmacology and experimental therapeutics. 2003;304(2):845-54.

51. Chen J, Luo J, Zhao Y, Pu L, Lu X, Gao R, et al. Increase in transgene expression by pluronic L64-mediated endosomal/lysosomal escape through its membrane-disturbing action. ACS applied materials & interfaces. 2015;7(13):7282-93.

CHAPTER 4

AKT2-related Biological Pathway Characterization and Validation in Breast and Colon Cancer Stem Cells

The information presented in this chapter was partially published in the following publications:

1) P Gener, D Rafael, Y Fernández, J Sayos, D Arango, I Abasolo, M Videira, S Schwartz Jr., Cancer Stem Cells and Personalized Cancer Nanomedicine. Nanomedicine (Lond), 11(3):307-20, 2016.
2) P Gener, L Gouveia, G Sabat, D Rafael, N Fort, A Arranja, Y Fernández, R Prieto, J Sayos, D Arango, I Abasolo, M Videira, S Schwartz Jr., Fluorescent CSC models evidence that targeted nanomedicines improve treatment sensitivity of breast and colon cancer stem cells. Nanomedicine, 11(8):1883-92, 2015.
3) D Rafael, P Gener, J Seras, A Perez, L Pindado, H Florindo, D Arango, J Sayos, I Abasolo, Y Fernandez, M Videira, S Schwartz Jr., AKT2 silencing impairs malignancy of breast and colon Cancer Stem Cells (CSCs) and prevents reversion from non-CSC to a CSC phenotype, *Article in preparation*.

Table of contents

4.1. Introduction

As mentioned previously, in the last years, accumulating evidences suggest that cancer dormancy, treatment resistance, and metastatic growth are sustained by the presence of CSC within the tumor, which provides a reservoir of highly malignant cells that are able to cause tumor recurrence after the therapeutic regimen (1-4).

Despite the effectiveness of many chemotherapeutic agents and anticancer treatments in terms of apparent tumor regression, the problematic of drug resistance observed for CSC and the consequent tumor recurrence arise as an important barrier and a great challenge to tumors eradication. Thereby, other therapeutic strategies have emerged, in particular the gene therapy approach and/or its combination with conventional therapies. One of the most interesting potentialities regarding gene therapy is the RNAi technology (5-7). To be able to implement effectively this strategy into the clinical practice, it is of utmost importance the complete understanding of the biological pathways involved in tumor development and progression in order to select an adequate target gene to be silenced.

AKT2 is one of the three isoforms of the PKB family highly expressed in breast and colon cancer tumors, which appears generally associated with the acquisition of a malignant phenotype by cancer cells (8-10). AKT/PKB is a key regulator of various cell processes and its signaling outcome, similarly to what happens in all cancer-related molecular mechanism, is highly dependent on cellular background, cell type and phenotype. AKT2 may act via different signaling mechanisms being TWIST and mTOR the main effectors downstream of AKT2 (11-14). In the context of CSC, TWIST arose as an interesting cancer target due to its important and well-known role in tumor invasion, migration, dissemination and drug resistance (15, 16) Signaling cascades initiated by TWIST-mediated effects regulated by AKT2 or those involved in EMT through the decrease of E-cadherin expression, bring reasonability to the expectancy over the selective silencing of this oncogene as an effective strategy to reduce cell malignancy (12, 14). EMT, usually defined as the process during which cells loose epithelial traits and acquire a mesenchymal phenotype, is commonly accepted as the critical step for the acquisition of malignance in epithelial cancer cells and in some cases as an activator of the CSC phenotype (14, 17-21). At the molecular level, the EMT is mainly characterized by the downregulation of E-cadherin, an essential cell-cell adhesion protein whereas mesenchymal markers such as Vimentin and N-cadherin tend to gradually raise their expression (14, 22). Regarding the importance of EMT in cancer development and CSC activation, the targeting of EMT related signaling axis like AKT2/TWIST in order to revert the EMT process and restore the initial epithelial phenotype, appears to be a promising strategy in cancer gene therapy.

Similarly, PI3K/AKT2/mTOR signaling axis is also involved in acquisition of malignant phenotype mainly through the over-activation of the mTOR signaling, as a result of increased activity of PI3K or AKT. mTOR activity was found to be deregulated in many types of cancer including breast, prostate, lung, melanoma, bladder, brain, and renal carcinomas. Moreover, recent studies have shown the links between PI3K/AKT2/mTOR signaling pathway and CSC biology (13, 23-28).

Despite that AKT2 silencing was previously investigated in various tumor types, its precise function in CSC was not addressed yet. Here, in order to assess if also in the CSC population, the AKT2 knockdown is able to impair the increased cellular tumorigenic ability, we silenced the AKT2 expression using a siRNA against AKT2 in different cancer cells lines with a defined CSC population.

4.2. Materials and Methods

4.2.1. Materials

RPMI medium, PBS, and FBS were purchase from Lonza (Barcelona, Spain). McCoy's medium, DMEM F-12 medium, L-glutamine, non-essential amino acids, 10000 U/mL penicillin and 10000 µg/mL streptomycin, 0.25% Trypsin-EDTA, blasticidin and sodium pyruvate were purchased from Gibco (Life Technologies Ltd., Madrid, Spain). Lipofectamine® 2000, trypan blue, DAPI, and SYBR Green were purchased from Molecular Probes (Life Technologies Ltd., Madrid, Spain). Cell Lytic M reagent and protease inhibitor cocktail were obtained from Sigma-Aldrich Quimica (Madrid, Spain).

Primary antibodies against TWIST and actin were obtained from Abcam (Cambridge, UK) while the antibodies against AKT2, mTOR, and E-cadherin purchased from Cell Signaling Technology (Leiden, The Netherlands) and Invitrogen (Life Technologies Ltd., Madrid, Spain), respectively. HRP-conjugated secondary antibodies (anti-mouse P0447) and anti-rabbit (P0217) were purchased from Dako (Barcelona, Spain).

siRNAs were designed by Shanghai Gene Pharma (Shanghai, China). The sense siAKT2 sequence used was 5'-GCUCCUUCAUUGGGUACAATT-3', while a non-specific sequence 5'-UUCUCCGAACGUGUCACGUTT-3' was used as control (siC).

4.2.2. Cell Lines and Culture Conditions

Breast cancer cell lines MCF7, MDA-MB-231, MDA-MB-468, and SKBR3; and colon cancer cell line HCT8 were obtained from American Type Culture Collection (ATCC, LGC

Standards, Barcelona, Spain) and are examples of both mesenchymal and epithelial cell lines **(Table 4.1)**.

MDA-MB-468, MDA-MB-231 and HCT8 cells were cultured in RPMI medium supplemented with 10% FBS, 1% penicillin-streptomycin, 1% L-glutamine, 1% non-essential amino acids, and 1% of sodium pyruvate.

SKBR3 cells were cultured in McCoy's medium supplemented with 10% FBS and 1% penicillin-streptomycin, while MCF7 cells were cultured in DMEM F-12 medium supplemented with 10% FBS and 1% penicillin-streptomycin.

Blasticidin (0.5 mg/ml) was used as a selective antibiotic for ALDH1A1/tdTomato cell lines. All cell lines were maintained at 37ºC under a 5% CO_2 water saturated atmosphere. The medium was changed every other day and, upon confluence, cells were harvested from plates with a 0.25% trypsin-EDTA solution and further used in *in vitro* studies or frozen.

Table 4. 1. List of the cell lines used in this study and their main phenotype.

Name	ATCC® number	Type	Main Phenotype
MDA-MB-231	HTB 26™	Breast (Triple Negative)	Mesenchymal
MDA-MB-468	HTB-132™	Breast (Triple Negative)	Mesenchymal
MCF7	HTB-22™	Breast (ER+)	Epithelial
SKBR3	HTB-30™	Breast (HER2+)	Mesenchymal
HCT8	CCL-244™	Colon	Epithelial

4.2.3. Fluorescence-Activated Cell Sorting (FACS)

FACS was used to sort CSC and non-CSC subpopulations from a heterogeneous population of MCF7, MDA-MB-231 and HCT8 cells. For the cell sorting, a starting amount of $5x10^6$ cells were used. Cells were detached, and re-suspended in PBS supplemented with 10% FBS with DAPI (1 µg/mL) used for vital staining. Cells were sorted according to tdTomato and DAPI expression in a FACS Aria cell sorter (BD Biosciences, Madrid, Spain) (29). Sorted cells were collected in complete medium without antibiotic and used for posterior applications.

4.2.4. Cell Transfection

The siRNAs were transfected into cells using Lipofectamine® 2000 accordingly to the manufacture instructions in medium without antibiotics. The medium was changed 6 hours after the transfection and cells were harvested after further 72 hours.

4.2.5. RNA Extraction and Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from cells using the RNeasy Micro Kit (Qiagen, Madrid, Spain) and the obtained RNA was reverse transcribed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Madrid, Spain) according to the manufacturer instructions.

Table 4. 2. List of the primers used in the study and their sequences.

Oligo name	Oligo Sequence (5' to 3')
TWIST F	GCAGGGCCGGAGACCTAG
TWIST R	TTTTTAGTTATCCAGCTCCAGAGTCTCTA
E-Cadherin F	TACGCCTGGGACTCCACCTA
E-Cadherin R	CCAGAAACGGAGGCCTGAT
Vimentin F	CTCCGGGAGAAATTGCAGG
Vimentin R	AGACGTGCCAGAGACGCATT
N-Cadherin F	TCAAAGCCTGGAACATATGTGATG
N-Cadherin R	GTATACTGTTGCACTTTTTCTCGTACAA
hAKT2F	CAAGGATGAAGTCGCTCACACA
hAKT2R	GAACGGGTGCCTGGTGTTC
18s F	AGT CCC TGC CCT TTG TAC ACA
18s R	GAT CCG AGG GCC TCA CTA AAC
Actin 01F	CATCCACGAAACTACCTTCAACTCC
Actin 01R	GAGCCGCCGATCCACAC
GADPH F	ACCCACTCCTCCACCTTTGAC
GADPH R	CATACCAGGAAATGAGCTTGACAA
mTOR F	CCTTAACGTCATTCGAGTCTGTGA
mTOR R	TCACAAAGGACACCAACATTCC
p53 F	GAAGAAACCACTGGATGGAGAATATT
p53 R	CAGCTCTCGGAACATCTCGAA
Bcl-2 F	ACCTGCACACCTGGATCCA
Bcl-2 R	AGAGACAGCCAGGAGAAATCAAA
GSK3b F	CTGCTTCAACCCCCACAAAT
GSK3b R	GATGCAGAAGCAGCATTATTGG
AKT1 F	GGCTCCCCTCAACAACTTCTC
AKT1 R	ACATGGAAGGTGCGTTCGA

The cDNA reverse transcription product was amplified with specific primers **(Table 4.2)** by qPCR using the SYBR Green method. The reaction was performed in triplicate in a 7500 real time PCR system (Applied Biosystems, Madrid, Spain). Actin and S18 were used as

endogenous controls. The relative mRNA levels were calculated using the comparative Ct method (2e- ΔΔCt).

4.2.6. Protein Extraction and Western Blotting (WB)

Cell pellets were lysed with the Cell Lytic M reagent containing a protease inhibitor cocktail. Proteins in the crude lysates were quantified using the BCA Protein Assay (Pierce Biotechnology, Madrid, Spain) and 20 µg of whole-cell lysates were separated by SDS-PAGE and transferred onto nitrocellulose membranes (Merck Millipore, Madrid, Spain). Blots were probed using primary antibodies listed in **Table 4.3**. Proteins were detected using corresponding HRP-conjugated secondary antibodies (anti-mouse or anti-rabbit) and incubated as described in **Table 4.3**.

Table 4. 3. List of primary antibodies used in the study and their specifications. ON – overnight, RT – room-temperature.

Primary antibody	Host	Dilution	Incubation
AKT2	Rabbit	1:1000	ON, 4°C
TWIST	Mouse	1:500	ON, 4°C
mTOR	Rabbit	1:1000	ON, 4°C
Actin	Rabbit	1:1000	1 hour, RT

4.2.7. Proliferation Assay

Breast cancer cell lines were seeded into six-well plates at a density of $2.5X10^5$ cells per well and allowed to adhere overnight. The next day cells were transfected as described previously. For cell counting, cells were trypsinized and stained with trypan blue and viable cells were counted at times 0, 24, 48 and 72 hours after transfection.

4.2.8. Wound Healing Migration Assay

For these assay, the different cells were seeded into six-well plates and cultured until high confluence. The monolayer was “wounded” by scraping it with a sterile tip. The wells were washed twice with medium without serum before being left in complete culture medium. Immediately after do the wound, the cells were transfected as explained before. The healing

process was examined during the time with an optical microscope and microphotographs were obtained at 0, 24, 48 and 72 hours. Areas were measured with the Image J software and compared with the wound area at time 0, in order to evaluate the migration rate of cells.

4.2.9. Cell Transformation Assay (Anchorage-independent Growth Assay)

The anchorage-independent growth of the different breast cancer cell lines were assessed by the CytoSelect™ Cell Transformation Assay Kit (Cell Biolabs, San Diego, CA, USA). A semisolid agar media were prepared accordingly the manufacturer instructions being the complexes Lipofectamine-siAKT2 or Lipofectamine-siC added to each well. After 6-8 days of incubation the colonies were observed under the microscope and the viable transformed cells counted using trypan blue.

4.2.10. Invasion Assay

The invasiveness of MDA-MB-468 and SKBR3 cells was assessed using the CytoSelect™ Laminin Cell Invasion Assay Kit (Cell Biolabs, San Diego, CA, USA). The polycarbonate membrane inserts coated with a uniform layer of Laminin I are used to discriminate invasive cells from noninvasive cells. The invasive cells are able to degrade the laminin matrix layer and pass through the pores of the polycarbonate membrane, clinging to the bottom of the insert membrane, while the noninvasive cells stay in the upper chamber. Briefly, the inserts were placed in a 24-well plate and a cell suspension containing $1X10^6$ cells/ml, previously transfected with the siAKT2 and the siC, was added to the insert. After 48 hours incubation, the invasive cells were dissociated from the membrane, lysed and quantified using the CyQuant® GR Fluorescent Dye (Cell Biolabs, San Diego, CA, USA).

4.2.11. Statistical Analysis

All the assays were run at least in triplicate, each involving at least two technical replicates. All results are expressed as the mean±SD. Statistical analysis was performed in Microsoft Office Excel™ 2007 using the unpaired Student's t-test. Differences were regarded as statistically significant when p-value were smaller than 0.05.

4.3. Results

4.3.1. AKT2 Silencing Impairs Proliferation and Migration of Cancer Cells

In order to determine the consequence of AKT2 inhibition on cell malignance, we investigate its effect in various cell lines (MDA-MB-231, HCT8, MCF7, MDA-MB-468, SKBR3). We confirmed the specificity of the siAKT2 sequence used, since 80% reduction of AKT2 expression was achieved and no reduction of AKT1 isoform was observed **(Figure 4.1A)**. The downregulation of AKT2 was confirmed also at the intracellular protein level **(Figure 4.5D)**.The effects of AKT2 downregulation in the different cell lines was firstly assessed by the proliferation and the migration assays. We observed a significant impairment of cell proliferation in MDA-MB-231, HCT8, MDA-MB-468 and SKBR3 transfected with the siAKT2 in comparison with the cells transfected with the siC or with the untreated cells. On the contrary, no effect in proliferation was observed after the AKT2 silencing in MCF7 cells **(Figure 4.1B)**. Similar results were observed for the migration capacity after AKT2 silencing, particularly in MDA-MB-231, MDA-MB-468 and SKBR3 cells where the migration rates were significantly impaired. Again, no changes on migration were observed in MCF7 cells **(Figure 4.1C)**. HCT8 showed just minimal migration ability and was thus excluded from the analysis (data not shown).

4.3.2. AKT2 Inhibits Anchorage-independent Growth and Invasion of CSC

After the functional assessment of the AKT2 silencing effect in parental cells, the behavior of the CSC fraction isolated from three different cell lines (MDA-MB-231, HCT8, MCF7) was investigated, after being transfected with siAKT2. CSC were isolated from the parental cells population by FACS based on the stable expression of ALDH1A1/tdTomato reporter vector. Only CSC are able to express tdTomato (red fluorescence) since its expression is driven by CSC specific promoter (ALDH1A1), while the non-CSC are not detected as red fluorescent cells in the total cell culture mixture (29). The CSC nature of tdTomato+ cells was previously confirmed by expression of stemness markers, tumorspheres formation and high *in vivo* tumorigenic capacity (29).

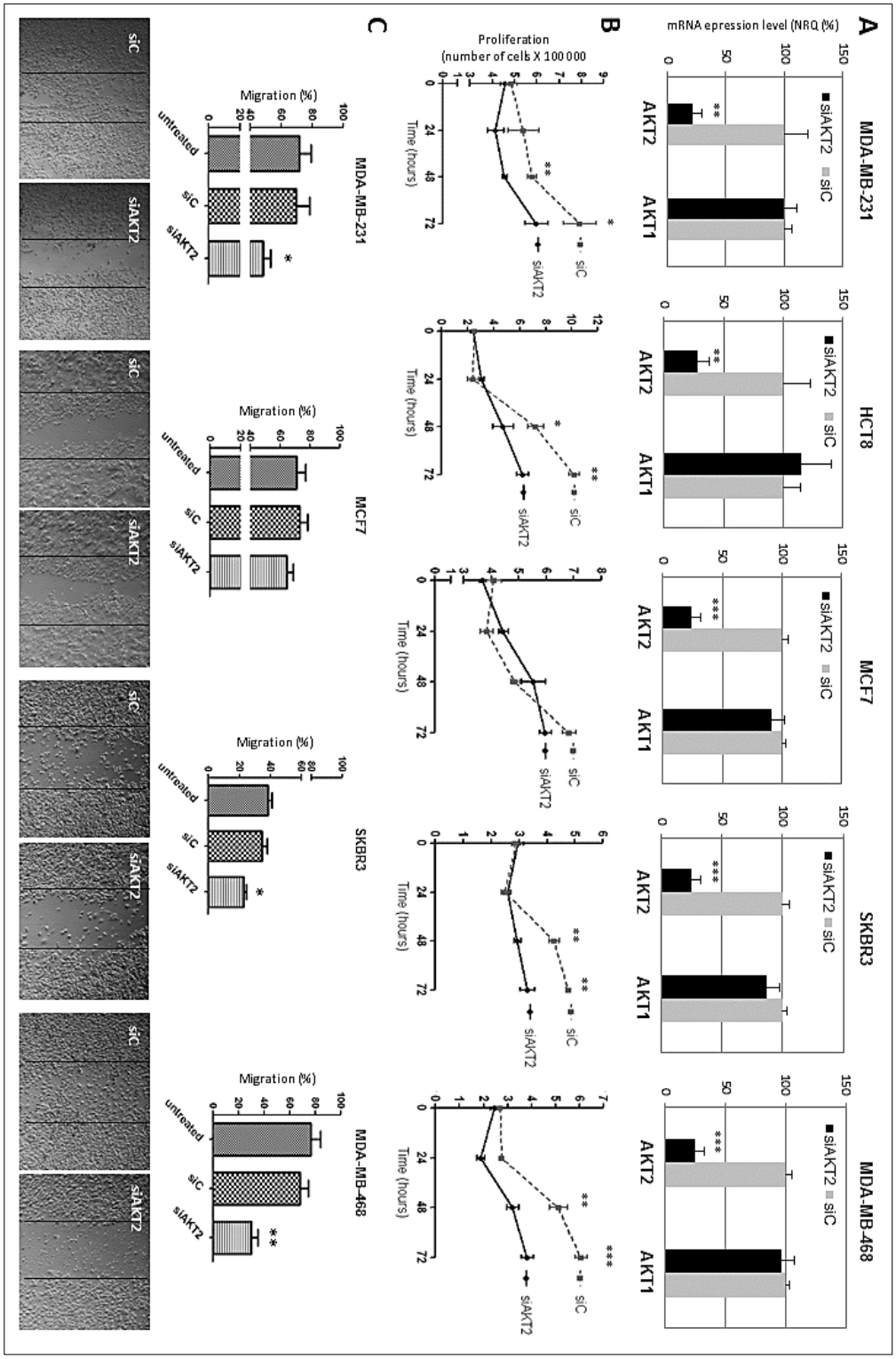


Figure 4. 1. Effect of AKT2 silencing in breast and colon cancer cell lines. A: qPCR - quantification of AKT2 and AKT1 expression levels in the cells transfected with the siAKT2 and siC; B: Proliferation assay – Differences in cell growth pattern between siAKT2 and siC treated cells; C: Migration assay – The images and the graphics compare the number of migratory cells after treatment with siAKT2 and siC. Results are expressed as mean±SD (n≥3) (* p< 0.05; ** p<0.01, ***p<0.001).

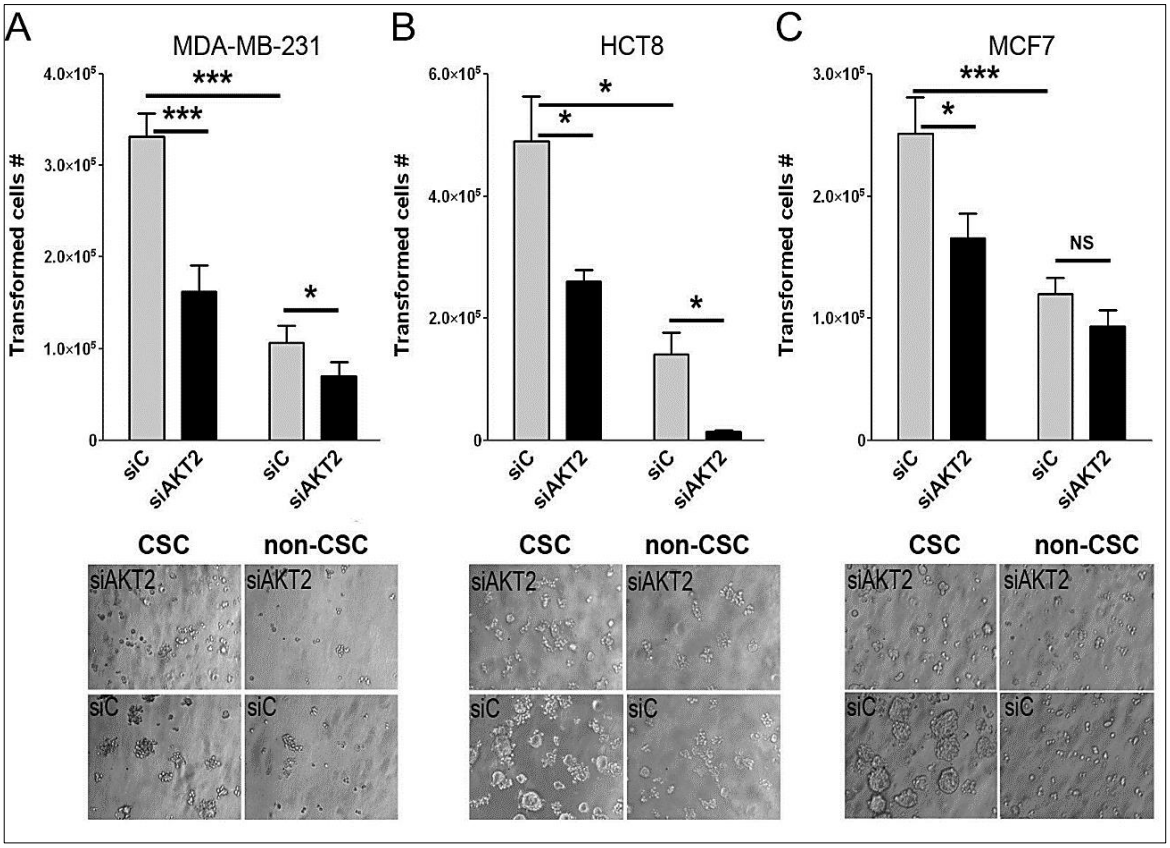


Figure 4. 2. siAKT2 transfection reduces cells transformation ability of MDA-MB-231 (A), HCT8 (B) and MCF7 (C) CSC and non-CSC. The graph represents the number of transformed cells comparing CSC and non-CSC transfected with siAKT2 and siC. The images are photographs of the colonies formed after 7 days of incubation with siAKT2 and siC. Results are expressed as mean±SD (n≥3) (* p< 0.05; ** p<0.01, ***p<0.001).

The most accurate and stringent *in vitro* assay for detecting malignant transformation of cells is the assessment of their capacity for anchorage-independent growth. Malignant transformation occurs via a series of genetic and epigenetic alterations that allow the cell population to proliferate independently of both external and internal signals that normally restrain growth. Generally, transformed cells have lower requirements for extracellular growth and is not restricted by cell-cell contact (30). We investigated the anchorage-independent growth capacity in fraction of non-CSC and CSC from three different cell lines: MDA-MB-231, HCT8, and MCF7. We observed significantly higher capacity of anchorage-independent growth in fraction of CSC sorted from all investigated cell lines compared to non-CSC, which confirmed the CSC nature of tdTomato+ cells **(Figure 4.2)**. Upon siAKT2 transfection the colonies formation ability was reduced predominantly in the CSC fraction of MDA-MB-231, HCT8 and MCF7. These results can be further confirmed by the significant decrease in the number of transformed cells. The number of transformed cells also decrease for the MDA-MB-231 and HCT8 non-CSC after transfection with the siAKT2; however no changes was observed in MCF7 non-CSC **(Figure 4.2).** Significant reduction

of transformation capacity after AKT2 silencing was also observed in MDA-MB-468 and SKBR3 cell lines, that are CSC enriched cells with high expression of ALDH1A1 **(Figure 4.4A).**

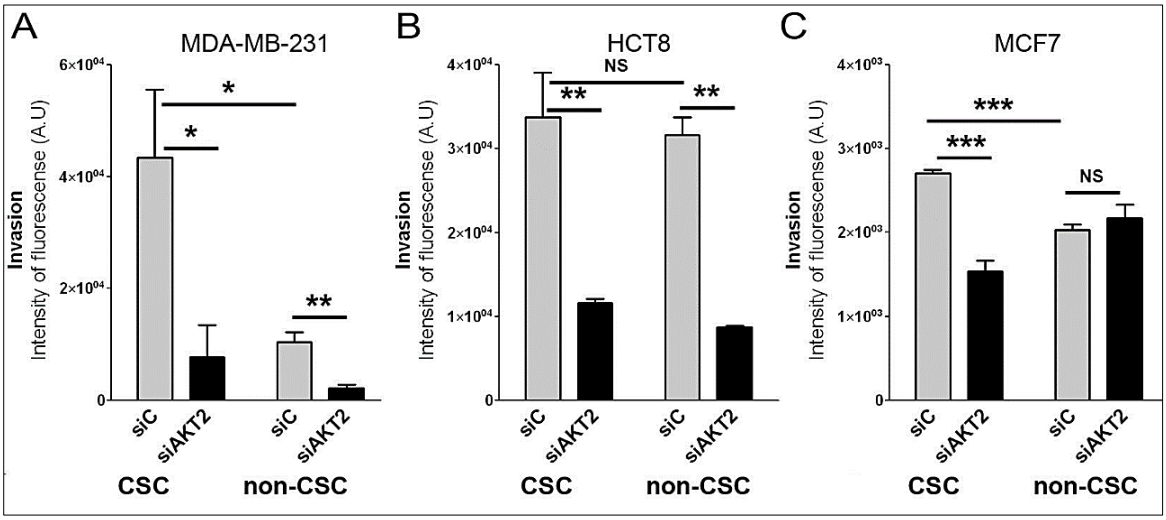


Figure 4. 3. Effects of siAKT2 in cells invasive capability of MDA-MB-231 (A), HCT8 (B) and MCF7 (C) CSC and non-CSC. The graph represents the number of cells that presented invasive capacity comparing CSC and non-CSC transfected with siAKT2 and siC. Results are expressed as mean±SD (n≥3) (* p<0.05; ** p<0.01, ***p<0.001).

Next, we investigated effect of siAKT2 transfection in the invasive potential of CSC. The capacity of malignant tumor cells to invade the normal surrounding tissues largely contributes to the cancers associated morbidity and mortality. Invasiveness is related with EMT process and involves several distinct cellular functions including adhesion, motility, and detachment (31). Worthy to note, is that CSC from MDA-MB-231 and MCF7 cell lines shown to be significantly more invasive than non-CSC. No significant differences were observed in the invasive potential of HCT8 CSC versus non-CSC. Importantly, in all cases the invasiveness of CSC was significantly reduced after AKT2 inhibition **(Figure 4.3)**. This behavior was also observed in MDA-MB-231 and HCT8 non-CSC. Even though CSC fraction from MCF7 cells showed a significant impairment of invasion after AKT2 silencing, no effect was observed in MCF7 non-CSC **(Figure 4.3)**. Moreover, the silencing of AKT2 promoted a significant reduction of invasion capacity in CSC enriched MDA-MB-468 and SKBR3 cell lines **(Figure 4.4B)**.

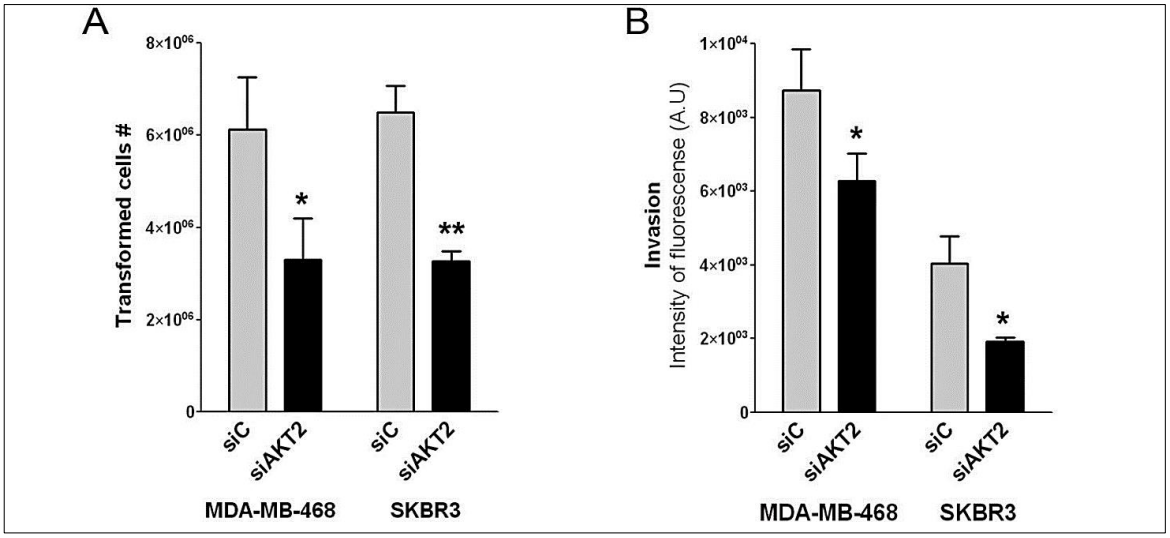


Figure 4. 4. Effects of siAKT2 in cells transformation ability and invasive capability of MDA-MB-468 and SKBR3. Graphs represent the number of transformed cells (A) and number of cells that presented invasive capacity (B) comparing CSC and non-CSC transfected with siAKT2 and siC. Results are expressed as mean±SD (n≥3) (* $p<0.05$; ** $p<0.01$, ***$p<0.001$).

4.3.3. AKT2 Inhibition is Achieved via Diverse Signaling Pathways

As mentioned previously, TWIST/AKT2 signaling is important for EMT regulation. Interestingly, CSC tend to have a more mesenchymal phenotype when compared to bulk tumor cells. Accordingly, CSC sorted from cell lines with epithelial phenotype (MCF7, HCT8) presented lower levels of the epithelial marker E-cadherin and higher levels of typical cell mesenchymal markers such as Vimentin and N-cadherin **(Figure 4.5B)**. Similarly, MDA-MB-468 and SKBR3, mesenchymal cell lines with high expression of ALDH1A1, also present low expression of E-cadherin and high expression of N-cadherin and Vimentin **(Figure 4.5C)**.

In this context, we investigated the expression of TWIST and mTOR in all studied cell lines. Despite the constant presence of AKT2 and mTOR (mRNA, protein), we found differences in terms of TWIST expression, being this transcription factor positively expressed in MCF7, MDA-MB-468 and SKBR3, while MDA-MB-231 and HCT8 shown to be TWIST negative ($TWIST^-$) cells **(Figure 4.5A and D)**. As expected after the silencing of AKT2, TWIST and the mTOR are downregulated **(Figure 4.5D)**.

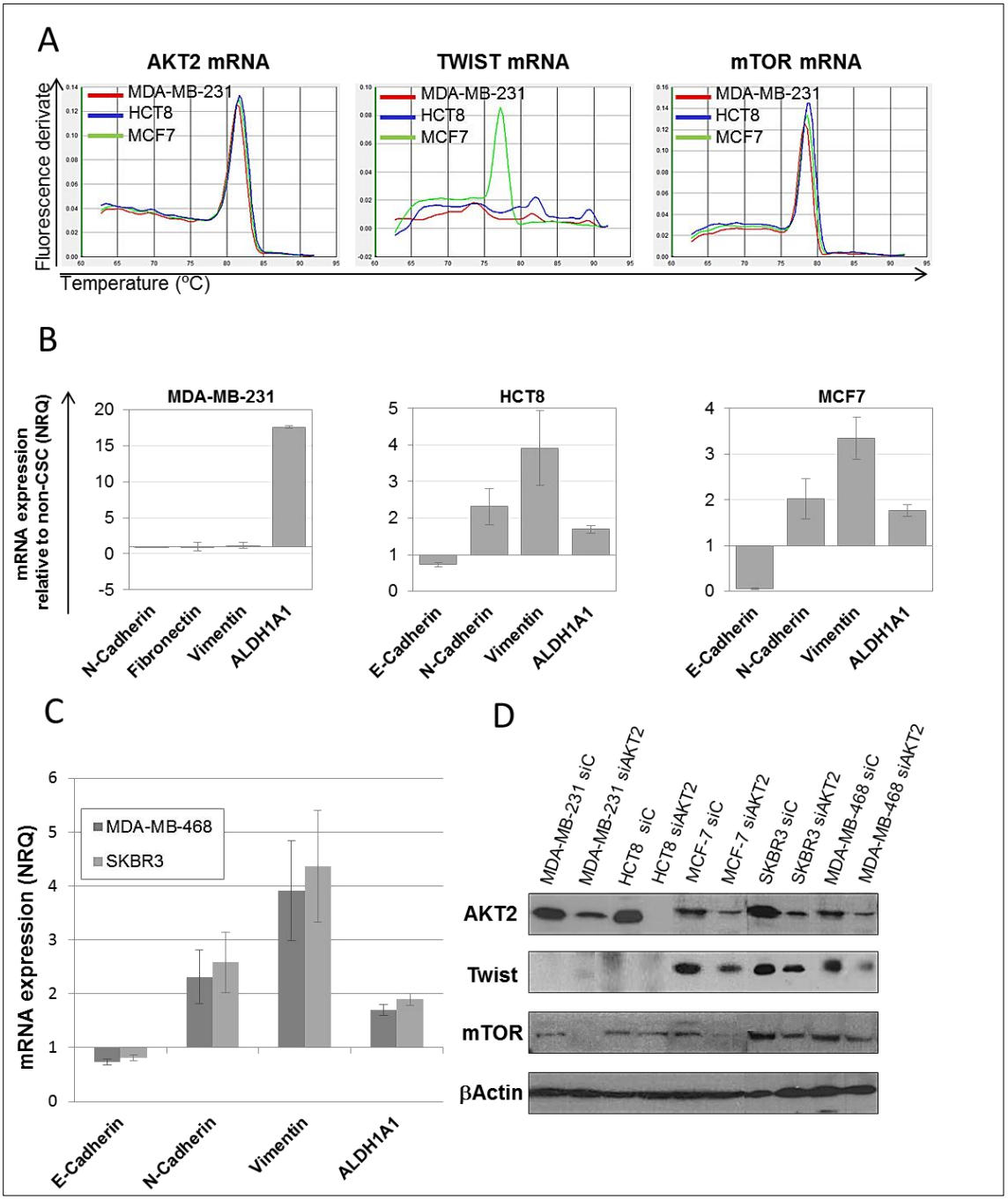


Figure 4. 5. Cell lines phenotypes based in the EMT/stemness markers. A) Amplification plot for TWIST, mTOR and AKT2 expression for MDA-MB-231, HCT-8 and MCF7 cells. B) qPCR quantification of several genes expression levels for MDA-MB-231, HCT-8 and MCF7 CSC. The presented values are relative to their non-CSC population. C) qPCR quantification of several genes expression levels for MDA-MB-468 and SKBR3 cells. D) Western Blot for AKT2, TWIST and mTOR protein expression levels in different cells after the treatment with siAKT2 and siC. β-actin was used as the housekeeping gene. Results are expressed as mean±SD (n≥3).

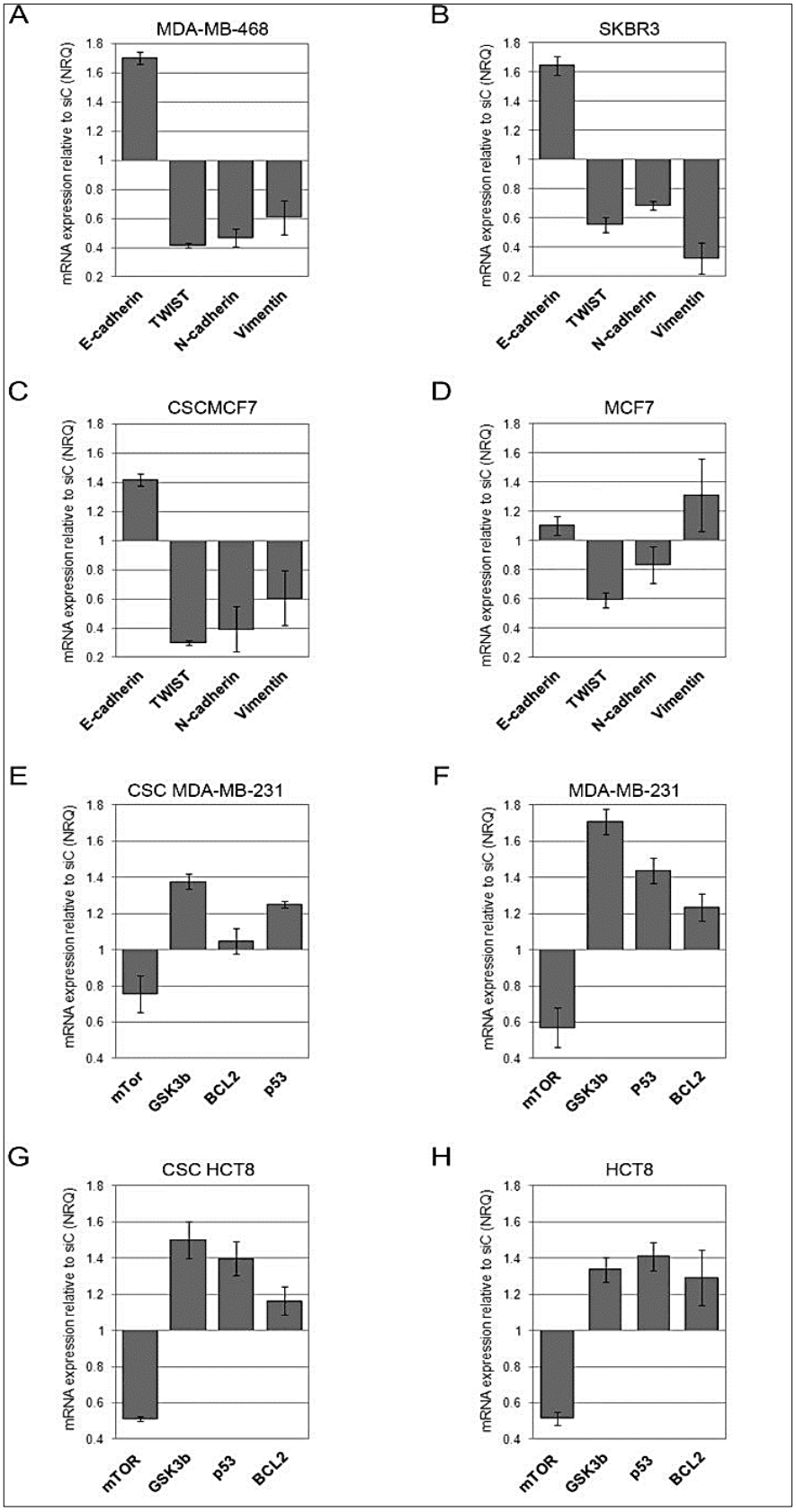


Figure 4. 6. Impact of AKT2 silencing in key regulators of EMT reversion and mTOR-dependent signaling pathways. Expression levels of different genes were quantified by qPCR in different cell lines after the treatment with siAKT2 and siC. Results are expressed as mean±SD (n≥3).

Accordingly, we observed MET gene expression signature in cell lines expressing TWIST (MDA-MB-468, SKBR3) after AKT2 silencing like a decrease in the expression of mesenchymal markers like Vimentin and N-cadherin. Notably, this trend is accompanied by cell restoration of E-cadherin expression, confirming our expectation that in this conditions cells will experiencing a switch from a mesenchymal to a more epithelial phenotype **(Figure 4.6A and B)**.

The observation of MET-related events in mesenchymal CSC isolated from epithelial MCF7 cell line upon AKT2 silencing, also related with the restoration of E-cadherin and further decrease in N-cadherin and Vimentin expression, stresses this players interchangeability triggered by this knockdown strategy **(Figure 4.6C)**. Even though we observed a downregulation of TWIST after AKT2 silencing, EMT reversion it was not observed in the case of non-CSC MCF7 epithelial cell line **(Figure 4.6D)**.

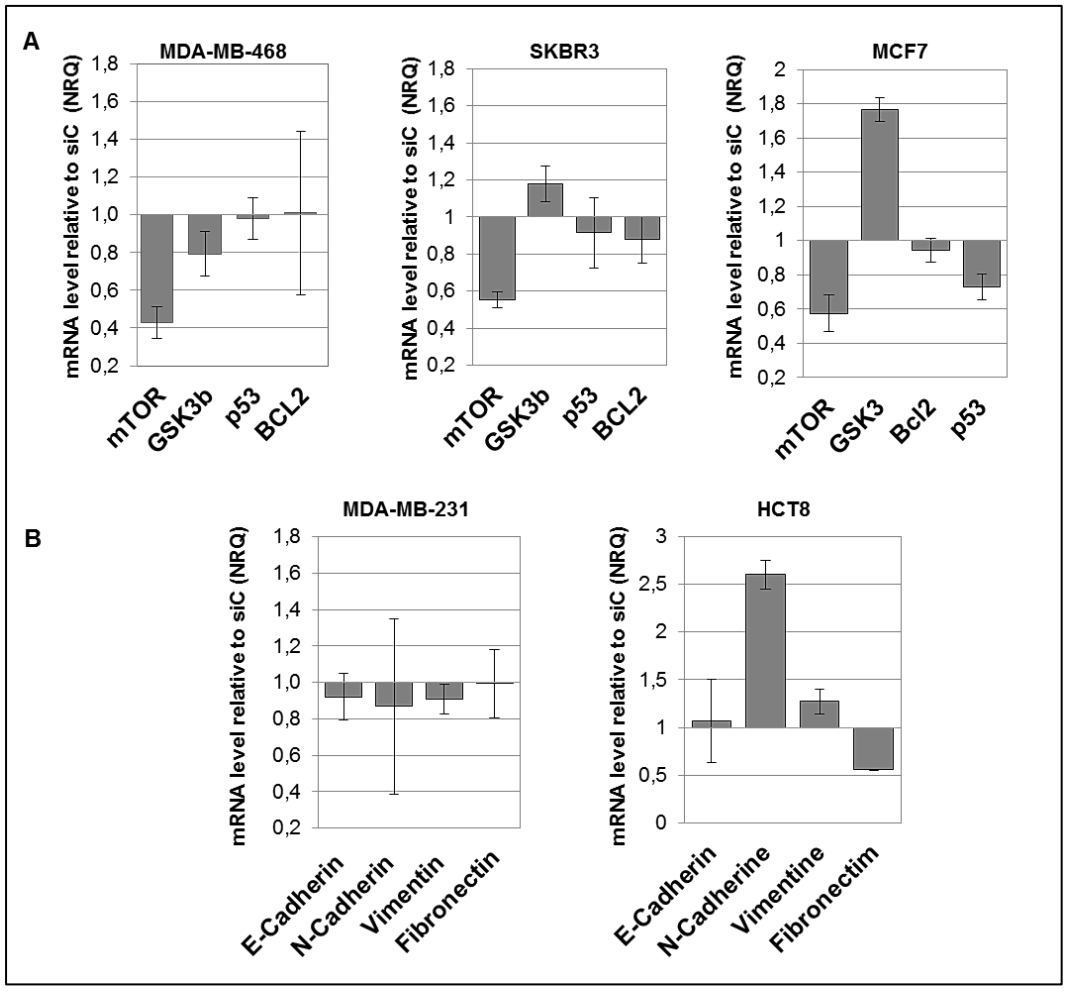


Figure 4. 7. Effects of AKT2 silencing in different signaling pathways. A) Efects of AKT2 silencing in the mTOR pathway for TWIST^{+} cells. B) Efffects of AKT2 silencing in the EMT reversion for TWIST^{-} cells. Results are expressed as mean±SD (n≥3).

In accordance with our proposed mechanism, TWIST$^-$ cells (MDA-MB-231, HCT8) didn't show the typical MET gene signature **(Figure 4.7B)**.

Although AKT2-TWIST cross talk is a key event for malignancy, in non-expressing TWIST cell lines the observed decrease in CSC malignance upon AKT2 silencing was related predominantly by the downregulation of mTOR. The essential contribution of mTOR for the regulation of cell growth arrest and inhibition of apoptosis was sustained by the finding that in response to the intracellular decrease of AKT2, was detected a constant decrease of mTOR expression and increase in GSK3b and p53 expression. This was observed in MDA-MB-231 and HCT8 cells **(Figure 4.6F and H)**. Similar results were observed in the fraction of sorted MDA-MB-231 CSC and HCT8 CSC **(Figure 4.6E and G)**. As expected, for the TWIST expressing cells, the downregulation of mTOR after AKT2 silencing didn't produce the mTOR-related effects in GSK3b, p53 and Bcl-2 **(Figure 4.7A)**.

The above described findings highlight that AKT2 silencing lead to the decrease of cell migration, proliferation, invasion and transformation via different signaling axis depending on the presence or absence of TWIST expression **(Figure 4.8)**. Regardless the signaling pathway, AKT2 silencing affects CSC malignancy and should be considered as prospective for successful anti-cancer therapies.

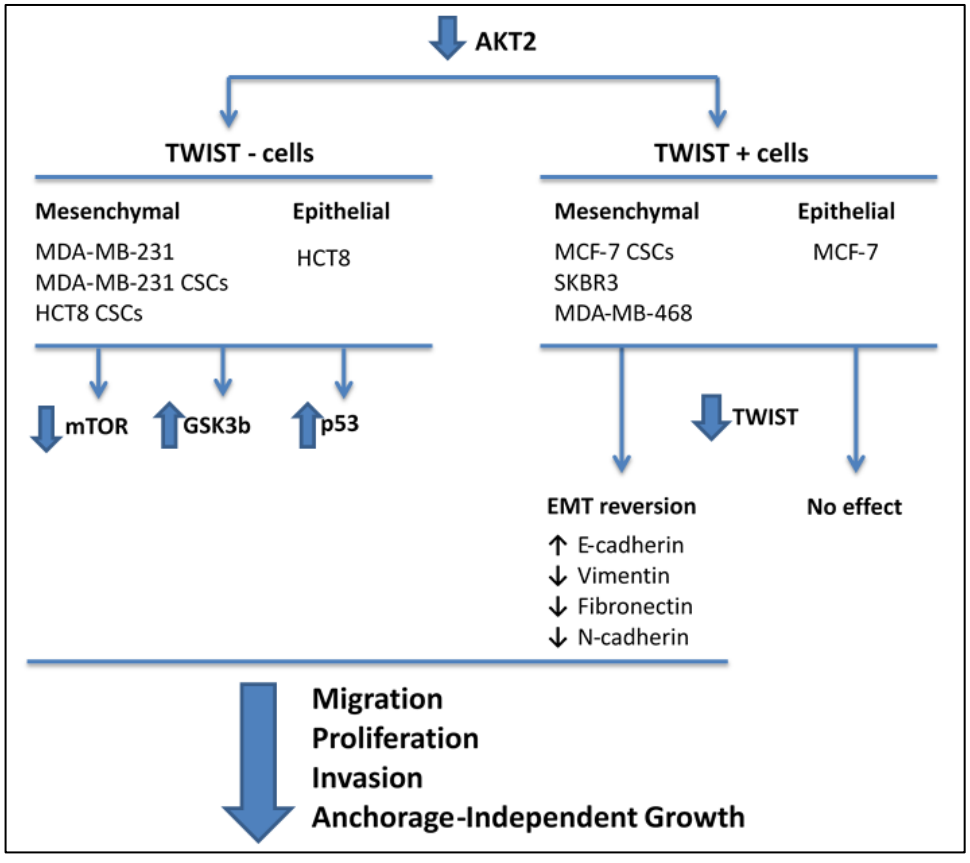


Figure 4. 8. Summary of AKT2 silencing effects in cancer development in the different cell lines, accordingly their phenotype and TWIST expression level.

4.4. Discussion

Due to their unique phenotype, CSC are resistant to the conventional chemo- and radiotherapy clinically practiced nowadays (4, 32). In addition, CSC are gaining relevance as triggering agents of cancer progression and malignancy, showing higher invasive capability than their non-CSC counterpart. Hereupon, even though several new promising anti-cancer treatments have been proposed over the past years, just few of them were extensively studied regarding their therapeutic efficacy also in population of CSC. Moreover, several reports have shown that if a therapy is not effective against CSC, the reservoir of resistant CSC will cause recurrence of aggressive tumor and metastatic growth over the time (33, 34). Study specifically the silencing effects of some genetic players like AKT2 in the CSC subpopulation is thus essential to develop new therapies that could impact in the clinical treatment of cancer.

In this study, a CSC model based on the tdTomato vector expression under the control of CSC specific ALDH1A1 promoter was used in order to study the efficacy of AKT2 silencing in CSC fraction. This CSC model was previously extensively validated both *in vitro* and *in vivo* (29). Moreover, here we observed higher anchorage-independent growth ability of tdTomato$^+$ CSC than the tdTomato$^-$ non-CSC, which further confirms this population malignant nature **(Figure 4.2)**. Importantly, after effective AKT2 silencing, the transformation and invasion capacity in all studied CSC fractions (MDA-MB-231, MCF7, HCT8) was significantly reduced. These results suggest that AKT2 silencing strategy may be successful also in fraction of highly aggressive CSC **(Figure 4.2 and 4.3)**.

AKT2 silencing also decreased the proliferation and migration capacity of various breast and colon cancer cell lines, such as MDA-MB-231, SKBR3, MDA-MB-468 and HCT8, with the remarkable exception of bulk MCF7 cells. No differences in terms of proliferation, migration, invasion and anchored independent growth capacity were observed in MCF7 bulk cells **(Figure 4.1)**. Of note, these were the only epithelial cells presenting expression of TWIST used in this study. Active AKT2/TWIST signaling axis leads to the acquirement of more mesenchymal phenotype. Contrary, AKT2 silencing confers to the cells more epithelial phenotype triggering MET (21). Since MCF7 cells regularly shown an epithelial phenotype with high expression of E-cadherin, the MET triggered via AKT2 silencing had no effect on bulk MCF7 cell population. On the other hand, CSC subpopulations isolated from MCF7 epithelial cells present a more mesenchymal phenotype than the non-CSC, with a decreased E-cadherin expression and an increased Vimentin and N-cadherin expression **(Figure 4.5B)**. In this mesenchymal CSC fraction (1-2%) isolated from epithelial MCF7 cells we observed a significant effect after AKT2 silencing in terms of reduced transformation

and invasion capacity **(Figure 4.2 and 4.3)**. Owing these cells involvement in tumor growth and maintenance, its reduction would suppose an important strike for further tumor progression. We observed similar results in the TWIST$^+$ MDA-MB-468 and SKBR3 mesenchymal cell lines that are also characterized by their high levels of ALDH1A1 expression, conferring them CSC-like properties (35) **(Figure 4.4)**. MET process after AKT2 silencing was confirmed by the genetic expression profile in mesenchymal TWIST$^+$ cell lines. We demonstrated that the AKT2 silencing is able to restore the levels of E-cadherin and decrease the levels of TWIST and other EMT markers like N-Cadherin, Vimentin and Fibronectin **(Figure 4.6A-C)**. In unsorted MCF7 cells, the only gene affected after AKT2 knockdown was TWIST, confirming the direct cross talk between AKT2 and TWIST, previously reported by Cheng GZ, *et al.* (2007) (11, 12) **(Figure 4.6D)**. Mani SA, *et al*. (2008), previously demonstrated a tight relationship between EMT activation and CSC in breast cancer. It was shown, that the ectopic expression of several EMT inducers (TWIST, Snail, TGF-β) triggers the expression of stem cell markers (CD44+ and CD24-) and enhances tumor development (36). Furthermore, EMT was observed also *in vivo* in CD8T immune cells which secrete EMT-inducing factors such as the TGF-β, presenting the resultant tumors characteristics of breast CSC (CD44+ and CD24-) (37).

In our study, CSC isolated from the TWIST$^-$ epithelial cell line, HCT8, shown to be more mesenchymal than the non-sorted bulk cells, with higher expression of N-cadherin and Vimentin and lower expression of E-cadherin **(Figure 4.5B)**. Despite the clear effect on the transformation and invasion capacity after AKT2 silencing in these cells, we did not observed MET phenotype in HCT8 CSC fraction **(Figure 4.7B)**. However, contrary to MCF7 epithelial cell line, we observed a decrease in the proliferation, invasion and transformation capacity of HCT8 tumor cells after AKT2 silencing **(Figure 4.1, 4.2 and 4.3)**. Moreover despite the reduction of the aggressiveness of both sorted CSC and unsorted cells, we have not observed any effect on EMT genes expression in other mesenchymal cell lines with TWIST$^-$ background (MDA-MB-231) **(Figure 4.7B)**. Taking into account these results, we conclude that for TWIST$^-$ cells, the AKT2 silencing is able to impair invasion and colony formation via a different pathway. We observed the blockage of the PI3/AKT2/mTOR signaling and consequent increase of GSK3b and pro-apoptotic genes such as p53 in both CSC and non-CSC subpopulations of mesenchymal TWIST$^-$ cells **(Figure 4.6E-H)**.

The novelty of this work lies in the comparison between CSC and non-CSC regarding the AKT2 silencing effects and the observation that, specifically for CSC, this inhibition is able to reverse the EMT phenotype in terms of gene expression, protein expression, migration, proliferation, anchorage-independent growth and invasion ability, being special interesting

this effect in the CSC population that usually present resistance against the conventional treatments.

4.5. Conclusions

The biggest challenge in cancer treatment is tumor recurrence and resistance. Both phenomena are sustained by the presence of CSC within the tumor. In this context, the potential of new targeted therapies should be investigated especially in fraction of CSC to provide its real probability to succeed in clinics. Since the RNAi therapy demonstrated important prospect in targeted cancer treatment, we investigated the efficiency of siAKT2 in CSC isolated from breast and colon cancer cell lines with different phenotypes. The obtained results demonstrated that the AKT2 inhibition is effective in terms of reducing cells migration, invasion, and transformation via different mechanisms and are not dependent on the presence of TWIST in the tumor. In cell lines expressing TWIST, AKT2 inhibition acts via MET, while in those cells not expressing TWIST, AKT2 silencing hampers mTOR pathway and alters the expression of apoptotic genes such as the Bcl-2 and p53. Regardless the signaling pathway, AKT2 silencing affects CSC malignancy, reverting their mesenchymal phenotype, reducing cell invasion, inhibiting colony formation or inducing apoptosis, and should be thus validated in further pre-clinical studies. Due to the beneficial effect of AKT2 silencing in both bulk cells and CSC, the therapy with AKT2 could be used i) to revert the tumor to its primary stage for easier chirurgical removal, and/or ii) prevent the tumor recurrence through the inhibition of CSC tumorigenicity.

4.6. References

1. Charafe-Jauffret E, Ginestier C, Birnbaum D. Breast cancer stem cells: tools and models to rely on. BMC Cancer. 2009;9:202.
2. Dean M, Fojo T, Bates S. Tumour stem cells and drug resistance. Nat Rev Cancer. 2005;5(4):275-84.
3. Lagadec C, Vlashi E, Della Donna L, Meng Y, Dekmezian C, Kim K, et al. Survival and self-renewing capacity of breast cancer initiating cells during fractionated radiation treatment. Breast Cancer Res. 2010;12(1):R13.
4. Yu Y, Ramena G, Elble RC. The role of cancer stem cells in relapse of solid tumors. Front Biosci (Elite Ed). 2012;4:1528-41.

5. Deng Y, Wang CC, Choy KW, Du Q, Chen J, Wang Q, et al. Therapeutic potentials of gene silencing by RNA interference: Principles, challenges, and new strategies. Gene. 2014.

6. Videira M, Arranja A, Rafael D, Gaspar R. Preclinical development of siRNA therapeutics: towards the match between fundamental science and engineered systems. Nanomedicine. 2014;10(4):689-702.

7. Kurreck J. RNA interference: from basic research to therapeutic applications. Angewandte Chemie. 2009;48(8):1378-98.

8. Irie HY, Pearline RV, Grueneberg D, Hsia M, Ravichandran P, Kothari N, et al. Distinct roles of Akt1 and Akt2 in regulating cell migration and epithelial-mesenchymal transition. The Journal of cell biology. 2005;171(6):1023-34.

9. Arboleda MJ. Overexpression of AKT2/Protein Kinase Bb Leads to Up-Regulation of b1 Integrins, Increased Invasion, and Metastasis of Human Breast and Ovarian Cancer Cells. 2003.

10. Dillon RL, Marcotte R, Hennessy BT, Woodgett JR, Mills GB, Muller WJ. Akt1 and akt2 play distinct roles in the initiation and metastatic phases of mammary tumor progression. Cancer research. 2009;69(12):5057-64.

11. Cheng GZ, Zhang WZ, Sun M, Wang Q, Coppola D, Mansour M, et al. Twist is transcriptionally induced by activation of STAT3 and mediates STAT3 oncogenic function. The Journal of biological chemistry. 2008;283(21):14665-73.

12. Cheng GZ, Chan J, Wang Q, Zhang W, Sun CD, Wang LH. Twist transcriptionally up-regulates AKT2 in breast cancer cells leading to increased migration, invasion, and resistance to paclitaxel. Cancer research. 2007;67(5):1979-87.

13. Umemura S, Mimaki S, Makinoshima H, Tada S, Ishii G, Ohmatsu H, et al. Therapeutic priority of the PI3K/AKT/mTOR pathway in small cell lung cancers as revealed by a comprehensive genomic analysis. J Thorac Oncol. 2014;9(9):1324-31.

14. Rafael D, Doktorovová S, Florindo HF, Gener P, Abasolo I, Schwartz S, et al. EMT blockage strategies: Targeting Akt dependent mechanisms for breast cancer metastatic behaviour modulation. Curr Gene Ther. 2015;15(3):300-12.

15. Beck B, Lapouge G, Rorive S, Drogat B, Desaedelaere K, Delafaille S, et al. Different levels of Twist1 regulate skin tumor initiation, stemness, and progression. Cell Stem Cell. 2015;16(1):67-79.

16. Zhou M, Hou Y, Yang G, Zhang H, Tu G, Du YE, et al. LncRNA-Hh Strengthen Cancer Stem Cells Generation in Twist-Positive Breast Cancer via Activation of Hedgehog Signaling Pathway. Stem Cells. 2015.

17. Onder TT, Gupta PB, Mani SA, Yang J, Lander ES, Weinberg RA. Loss of E-cadherin promotes metastasis via multiple downstream transcriptional pathways. Cancer research. 2008;68(10):3645-54.

18. Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. The Journal of clinical investigation. 2009;119(6):1420-8.

19. Radisky DC, LaBarge MA. Epithelial-mesenchymal transition and the stem cell phenotype. Cell Stem Cell. 2008;2(6):511-2.

20. Saito T, Mimori K. [Cancer stemness and circulating tumor cells]. Nihon Rinsho. 2015;73(5):806-10.

21. Brabletz T. EMT and MET in metastasis: where are the cancer stem cells? Cancer Cell. 2012;22(6):699-701.

22. Katsuno Y, Lamouille S, Derynck R. TGF-β signaling and epithelial-mesenchymal transition in cancer progression. Curr Opin Oncol. 2013;25(1):76-84.

23. Bai H, Li H, Li W, Gui T, Yang J, Cao D, et al. The PI3K/AKT/mTOR pathway is a potential predictor of distinct invasive and migratory capacities in human ovarian cancer cell lines. Oncotarget. 2015;6(28):25520-32.

24. Chen J, Shao R, Li F, Monteiro M, Liu JP, Xu ZP, et al. PI3K/Akt/mTOR Pathway Dual Inhibitor BEZ235 Suppresses the Stemness of Colon Cancer Stem Cells. Clin Exp Pharmacol Physiol. 2015.

25. Ji Y, Di W, Yang Q, Lu Z, Cai W, Wu J. Inhibition of Autophagy Increases Proliferation Inhibition and Apoptosis Induced by the PI3K/mTOR Inhibitor NVP-BEZ235 in Breast Cancer Cells. Clin Lab. 2015;61(8):1043-51.

26. Patruno A, Pesce M, Grilli A, Speranza L, Franceschelli S, De Lutiis MA, et al. mTOR Activation by PI3K/Akt and ERK Signaling in Short ELF-EMF Exposed Human Keratinocytes. PLoS One. 2015;10(10):e0139644.

27. Piao Y, Li Y, Xu Q, Liu JW, Xing CZ, Xie XD, et al. Association of MTOR and AKT Gene Polymorphisms with Susceptibility and Survival of Gastric Cancer. PLoS One. 2015;10(8):e0136447.

28. Xia P, Xu XY. PI3K/Akt/mTOR signaling pathway in cancer stem cells: from basic research to clinical application. Am J Cancer Res. 2015;5(5):1602-9.

29. Gener P, Gouveia LP, Sabat GR, de Sousa Rafael DF, Fort NB, Arranja A, et al. Fluorescent CSC models evidence that targeted nanomedicines improve treatment sensitivity of breast and colon cancer stem cells. Nanomedicine. 2015;11(8):1883-92.

30. Creton S, Aardema MJ, Carmichael PL, Harvey JS, Martin FL, Newbold RF, et al. Cell transformation assays for prediction of carcinogenic potential: state of the science and future research needs. Mutagenesis. 2012;27(1):93-101.

31. Shaw LM. Tumor cell invasion assays. Methods Mol Biol. 2005;294:97-105.

32. Dragu DL, Necula LG, Bleotu C, Diaconu CC, Chivu-Economescu M. Therapies targeting cancer stem cells: Current trends and future challenges. World J Stem Cells. 2015;7(9):1185-201.

33. Nandy SB, Gangwani L, Nahleh Z, Subramani R, Arumugam A, de la Rosa JM, et al. Recurrence and metastasis of breast cancer is influenced by ovarian hormone's effect on breast cancer stem cells. Future Oncol. 2015;11(6):983-95.

34. Sehl ME, Shimada M, Landeros A, Lange K, Wicha MS. Modeling of Cancer Stem Cell State Transitions Predicts Therapeutic Response. PLoS One. 2015;10(9):e0135797.

35. Marcato P, Dean CA, Pan D, Araslanova R, Gillis M, Joshi M, et al. Aldehyde dehydrogenase activity of breast cancer stem cells is primarily due to isoform ALDH1A3 and its expression is predictive of metastasis. Stem Cells. 2011;29(1):32-45.

36. Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, Zhou AY, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell. 2008;133(4):704-15.

37. Santisteban M, Reiman JM, Asiedu MK, Behrens MD, Nassar A, Kalli KR, et al. Immune-induced epithelial to mesenchymal transition in vivo generates breast cancer stem cells. Cancer Res. 2009;69(7):2887-95.

CHAPTER 5

Functional Validation of Amphiphilic-based Polymeric Micelles for siRNA Delivery and Cancer Stem Cells Genes Inhibition

The information presented in this chapter was partially published in the following publication:

1) D Rafael, P Gener, F Andrade, J Seras, S Montero, Y Fernandez, I Abasolo, S Schwartz Jr., M Videira, Functional Validation of Amphiphilic-Based Polymeric Micelles for siRNA Delivery and Cancer Stem Cells Genes Inhibition, *Article in preparation*.

Table of contents

5.1. Introduction

The major challenge regarding the RNAi bases therapies is the delivery of OGN to the cells without suffering premature degradation by serum nucleases. In this context, PM for siRNA delivery were design based on amphiphilic block-co-polymers previously developed within the group (1). Pluronic® polymers were chosen due to their recognized ability to enhance the transfection efficiency of genetic material, and their PEGylated surface, offering stealth properties to the system (2).

Based on the compounding and formulation development studies presented in **Chapter 3**, here is proposed a new system for siRNA delivery that combines i) polyplexes spontaneously formed by electrostatic interaction between the negatively charged siRNA molecules and the positively charged chains of 10K branched PEI, with ii) Pluronic® F127-based PM formed by FH technique.

To access the biological efficacy of these PM we firstly produced the micelles using a siRNA against GFP in breast cancer cells, which obtained a gene silencing higher than 80%, the same or even greater than the gold standard vector Lipofectamine® 2000. After we produced our micelles with the siRNA against AKT2 and tested them in breast CSC isolated from two different cell lines. With these micelles we were able to observe a reduction of the CSC invasion and transformation capacities.

5.2. Material and Methods

5.2.1. Materials

Pluronic® F127 was kindly provided by BASF (Ludwigshafen, Germany), while 10k branched PEI was purchased from Alfa Aesar (Thermo Fisher GmbH, Karlsruhe, Germany). DMSO, MTT, 5-DTAF were from Sigma-Aldrich (Madrid, Spain), while sodium bicarbonate and paraformaldehyde (PFA) were obtained from Merck KGaA (Darmstadt, Germany). RPMI medium, PBS, and FBS were purchase from Lonza (Barcelona, Spain). L-glutamine, non-essential amino acids, 10000 U/mL penicillin and 10000 µg/mL streptomycin, 0.25% Trypsin-EDTA, blasticidin and sodium pyruvate were purchased from Gibco (Life Technologies Ltd., Madrid, Spain). siRNA-GFP positive and negative control, Lipofectamine® 2000, LysoTracker® Red DND-99, DAPI, SYBR Green, and ProLong® Gold Antifade Mountant were purchased from Molecular Probes (Life Technologies Ltd., Madrid, Spain). Angarose and TBE buffer (10X) were obtained Sigma-Aldrich (St. Louis, MO, USA). siRNAs were designed by Shanghai Gene Pharma (Shanghai, China); sense siAKT2

sequence used was 5'-GCUCCUUCAUUGGGUACAATT-3', while a non-specific sequence 5'-UUCUCCGAACGUGUCACGUTT-3' was used as control (siC).

Other reagents used are methanol, ethanol and acetic acid from analytical grade (Merck, Germany) and Type 1 ultrapure water (18.2 MΩ.cm at 25 ºC, Milli-Q®, Billerica, MA, USA).

5.2.2. Polyplexes PEI-siRNA Production

PEI-siRNA complexes were prepared by simple complexation by adding either the 10k PEI aqueous solution dropwise to an equal volume of siRNA aqueous solution followed by a 10 seconds vortex mixing before incubating them at room temperature for 30 minutes. The PEI-siRNA complexes were prepared at N/P ratio 50 calculated according to the previously described **Equation 3.2**.

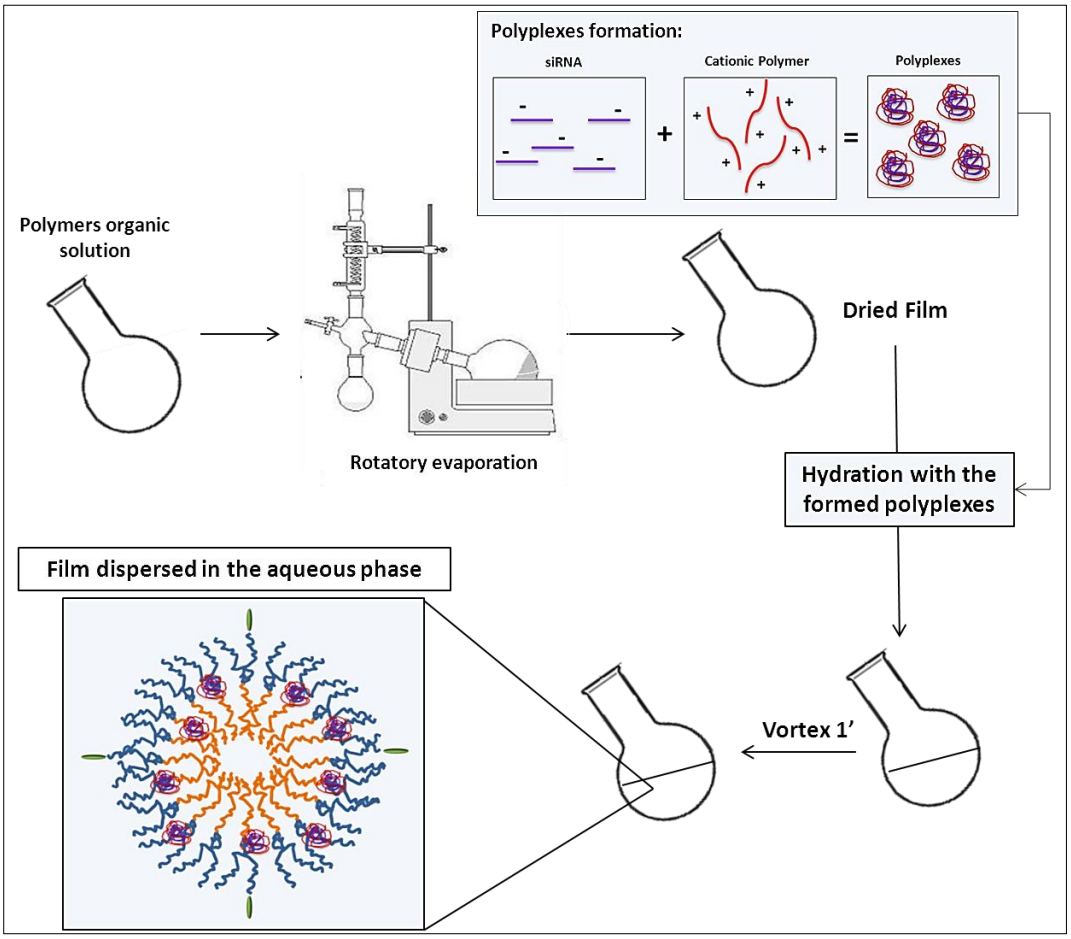


Figure 5. 1. Schematic representation of micelles production.

5.2.3. Production of Micelles

PM were prepared using the FH technique previously described (1). Briefly, the polymer was individually weighted and dissolved in a mixture of methanol:ethanol (1:1). Then, the solvent was removed under vacuum in a rotary evaporator and the formed film was left to dry overnight at room temperature to eliminate any remaining solvent. The film was then hydrated with the previously prepared polyplexes and vortexed during 1 minute **(Figure 5.1)**. The obtained dispersion was filtered through a 0.22 µm syringe filter for sterilization and remove possible aggregates.

5.2.4. Micelles Physicochemical Characterization

PM mean hydrodynamic diameter and polydispersity index were measured by DLS and zeta potential was assessed by laser doppler micro-electrophoresis using a NanoZS (Malvern Instruments, UK). Each type of formulation was produced and analyzed in triplicate.

The morphology of PM was assessed by TEM. For that, samples were placed on a grid, treated with uranil acetate and then observed in a JEM-1210 Transmission Electron Microscope (JEOL Ltd., Tokyo, Japan).

The encapsulation efficiency were assessed by quantification of the free siRNA in filtrates using a UV-Vis spectrophotometers (Nanodrop 2000, Thermo Scientific, USA) after filtration of the formulations by centrifugation for 10 minutes at 10,000 rpm and 4ºC, using 100k pore filters (Nanosep® Centrifugal Devices, Pall Corporation, Spain).

Gel electrophoresis was also performed to evaluate siRNA condensation. The PM were loaded onto 1% agarose gel with 6× loading buffer. The mixture was separated in 0.5× Tris/Borate/EDTA (TBE) buffer at 100V for 25 minutes. The siRNA bands were visualized using an ultra violet (UV) imaging system (Uvidoc, UVItec Ltd, Cambridge, UK).

The osmolality of formulations was determined at room temperature using a Micro-Osmometer M3320 (Advanced Instruments, Inc., MA, USA). Triplicates of each formulation were analyzed.

5.2.5. Cell Lines and Culture Conditions

MCF7 (ATCC number HTB-22) and MDA-MB-231 (ATCC number HTB-26) breast cancer cell lines were obtained from American Type Culture Collection (ATTC, LGC Standards,

Barcelona, Spain). The cells were cultured in RPMI medium supplemented with 10% FBS, 1% penicillin-streptomycin, 1% L-glutamine, 1% non-essential amino acids and 1% of sodium pyruvate. Blasticidin (0.5 mg/ml) was used as a selective antibiotic for ALDH1A1/tdTomato cell lines. All cell lines were maintained at 37ºC under 5% CO_2 saturated atmosphere. The medium was changed every other day and, upon confluence, cells were harvested from plates with 0.25% trypsin-EDTA to be passed to other plates to continue expansion, be frozen or used in *in vitro* studies.

5.2.6. Cell Transfection with the PM

The different PM-siRNA were transfected into cells accordingly **Table 5.1**. For silencing efficacy assays the medium was changed after 4 hours of incubation with the PM. For the toxicity assays the cells were left 24 hours in contact with the PM, while for the internalization assays the cells were incubated with the PM at several time points (from 3 seconds to 24 hours)

Cells were harvested 72 hours after the transfection. For the internalization experiments the particles were diluted 1:10

For some experiments, Lipofectamine® 2000 (Life Technologies, Madrid, Spain) were used as a transfection positive control accordingly the supplier protocol in order to obtain a final siRNA concentration in the well of 200 nM.

Table 5. 1. Transfection conditions accordingly the different experiments.

	Day 0: before transfection		**Day 1:** during transfection time (4 hours)		
Plates	**Cells seeded (cells/well)**	**Vol. of medium (µl)**	**Vol. of medium (µl)**	**Vol. of formulation (µl)**	**Final siRNA concentration (nM)**
96 well	$5.0x10^3$	100	50	50	200
24 well	$5.0x10^4$	1000	100	100	200
6 well	$2.0x10^5$	2000	250	250	200

5.2.7. Serum Stability Assay

To study the PM stability in serum, a special medium containing a concentration of FBS 50 % was prepared in order to predict the nanoparticles aggregation pattern *in vivo*. The PM were incubated with medium-FBS (50%) and samples were collected at different time-points (0, 4, 6, 12 and 24 hours) for DLS measurement.

5.2.8. Assessment of Micelles Toxicity

In order to assess the effect of formulations on the viability of breast cancer cell lines, 5000 cells/well were seeded in 96-well plates and left to attach for 24 hours. Then, cells were incubated with increasing concentrations of PM:siC, PEI, F127 and PEI:siC complexes for 24 hours. After this incubation time, the medium was changed and the cells left for 72 hours. Complete medium was used as negative control and 10% DMSO as positive control of toxicity. Cell viability was measured 72 hours upon transfection by MTT assay. The absorbance of each well was read on an absorbance microplate reader ELx800 (BioTek, Germany), at 590 nm. The results of cell viability were used for the determination of IC_{50} index by nonlinear regression of the concentration-effect curve fit using Prism 6.02 software (GraphPad Software, Inc., CA, USA).

5.2.9. Fluorescence-Activated Cell Sorting (FACS)

FACS was used to sort CSC and non-CSC subpopulations from a heterogeneous population of MCF7 and MDA-MB-231 cells. For the cell sorting, a starting amount of $5X10^6$ cells were used. Cells were detached with 0.25% trypsin-EDTA and resuspended in PBS supplemented with 10% FBS and DAPI (1 µg/mL) used for vital staining. Cells were sorted according to tdTomato and DAPI expression in a FACS Aria cell sorter (BD Biosciences, Madrid, Spain). Sorted cells were collected in complete medium without antibiotic and used for posterior applications.

5.2.10. Micelles Internalization

To assess the internalization behavior of PM in MCF7 and MDA-MB-231 cells, 5-DTAF-fluorescently labeled PM were used (conjugation method described in **Chapter 3**, **Figure 3.2**).

a) Confocal microscopy (qualitative analysis)

For confocal microscopy analysis, $2.5x10^5$ cells were cultured into glass coverslips coated with 0.1% gelatin in 6 well plates for 24 hours to allow adhesion. Then, cells were incubated for 4 hours with the labeled PM and after this time, the lysosomes were stained using the Lysotracker® Red during 30 minutes at 37 ºC. Subsequently, the cells were fixed in 4% PFA

at 4ºC for 30 minutes followed by nuclei staining with DAPI (0.2 mg/mL) for 5 minutes in the dark. Cells were viewed under a Spectral Confocal Microscope MFV1000 Olympus (Olympus, USA). The 561 nm excitation wavelength of the green laser (10mW) was used for selective detection of the red fluorochrome (Lysotracker® Red). The 488 nm excitation wavelength of Argon multiline laser (40 mW) was used for selective detection of the green fluorochrome (5-DTAF). The nuclear staining DAPI was excited at 405 nm with a violet laser (6 mW). Minimal single optical sections were collected for each fluorochrome sequentially. The generated images were merged and analyzed with the FV10-ASW 3.1 Viewer software (Olympus, USA) and exported as jpg image format.

b) Flow cytometry (quantitative analysis)

Flow cytometry analysis was used to verify internalization of nanoparticles in MDA-MB-231 and MCF7 ALDH1A1 tdTomato cells with around 50% of tdtomato+ cells. $2x10^5$ cells were seeded in complete medium in 96 well plates for 24 hours to allow adhesion. Micelles were added to cells at different time points: 1, 3, 10, 30 minutes and, 1, 2, 4, 6, 8 and 20 hours. Then, cells were washed with 1x PBS, detached with 0.25% trypsin-EDTA, and resuspended in PBS supplemented with 10% FBS and DAPI (1 µg/mL) used for vital staining. The plate was analyzed in a cytometer Fortessa (BD Biosciences, USA). Data was analyzed with FCS Express 4 Flow Research Edition software (De Novo Software, Los Angeles, USA). Contaminants were removed by forward and side scatter gating. For each sample, at least 10000 individual cells were collected and the mean fluorescence intensity was evaluated.

5.2.11. Green Fluorescent Protein Silencing Efficacy

MDA-MB-231 cells expressing GFP were used to assess the micelles silencing efficacy. Micelles prepared using a siRNA against the GFP were added to cells at a concentration of $5x10^4$ cells/ml in 96 well plates. For these test, as a positive control were used cells transfected with the complexes formed between the Lipofectamine ® 2000 and the GFP prepared accordingly to the supplier instructions. On the other hand, cells transfected in the same conditions with PM prepared with a siC were used as negative control. The expression of GFP in cells after transfection was assessed with a fluorescence microscope (Olympus, USA). A video of the cells during 72 hours after transfection was recorded using Motorized Fluorescent Microscope BX61 (Olympus, USA) in order to confirm GFP decreasing over

the time. Fluorescence intensity per cell over all the time of the experiment was quantified using the ImageJ 1.48v software (National Institutes of Health, MD, USA). The intensity of cells fluorescence was also measured using a Fluorescent Microplate reader FLX800 (BioTek, Germany).

5.2.12. RNA Extraction and Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from cells using the RNeasy Micro Kit (Qiagen, Madrid, Spain) and the obtained RNA was reverse transcribed with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Madrid, Spain) according to the manufacturer instructions. The cDNA reverse transcription product was amplified with specific primers for AKT2 (hAKT2 F: 5' CAA GGA TGA AGT CGC TCA CAC A 3'; hAKT2 R: 5' GAA CGG GTG CCT GGT GTT C 3') GADPH (hGADPH F: 5' ACC CAC TCC TCC ACC TTT GAC; hGADPH R: 5' CAT ACC AGG AAA TGA GCT TGA CAA 3') and Actin (hActin F: 5' CAT CCA CGA AAC TAC CTT CAA CTC C 3'; hActin R: 5'GAG CCG CCG ATC CAC AC 3') by qPCR using the SYBR Green method. The reaction was performed in triplicate on a 7500 Real time PCR system (Applied Biosystems, Madrid, Spain). Actin, GADPH, and S18 were used as endogenous controls. The relative mRNA levels were calculated using the comparative Ct method (2e- ΔΔCt).

5.2.13. Cell Transformation Assay (Anchorage-independent Growth Assay)

The anchorage-independent growth of the different breast cancer cell lines were assessed by the CytoSelect™ Cell Transformation Assay Kit (Cell Biolabs, San Diego, CA, USA). A semisolid agar media were prepared accordingly the manufacturer instructions prior addition of PM-siAKT2 or PM-siC to each well. After 6-8 days of incubation the colonies were observed under an optical microscope and the viable transformed cells counted using trypan blue.

5.2.14. Invasion Assay

Cells invasiveness was assessed using the CytoSelect™ Laminin Cell Invasion Assay Kit (Cell Biolabs, San Diego, CA, USA) as previously described in **Chapter 4**. Briefly, the inserts were placed in a 24 well plate and $2.5x10^4$ cells previously transfected (24 hours before) with the PM-siAKT2 and the PM-siC, were added to the upper chamber. After 48 hours

incubation, the invasive cells were dissociated from the membrane, lysed and quantified using the CyQuant® GR Fluorescent Dye using a fluorescent microplate reader FLX800 (BioTek, Germany).

5.2.15. *In vivo* Maximum Tolerated Dose (MTD) Determination

Female athymic nude mice (Hsd:Athymic Nude-Foxn1 nu/nu; Harlan Interfauna Iberica, Barcelona, Spain) were kept in pathogen-free conditions and used at 6 weeks of age. Animal care was handled in accordance to guidelines for the Care and Use of Laboratory Animals of the Vall d'Hebron University Hospital based on Federation of Laboratory Animal Science Associations (FELASA) recommendations and the European Union legislation (European Parliament and Council Directive 2010/63/EU). The experimental procedures were approved by the Animal Experimentation Ethical Committee of the institution.
Animals were randomly divided in 4 groups (n=3) and intravenously administered in the tail vein with PBS 1X (control group) or PM at doses of 20, 40 or 80 mg per kilogram of body weight (single administration). Body weight changes and side effects were monitored during 12 days post-administration. For side effects evaluation the reduction of voluntary movement, irregular breathing, motor disturbances, seizures, sudden death, flushed skin, lacrimation, dehydration and hunched position was monitored.

5.2.16. Statistical Analysis

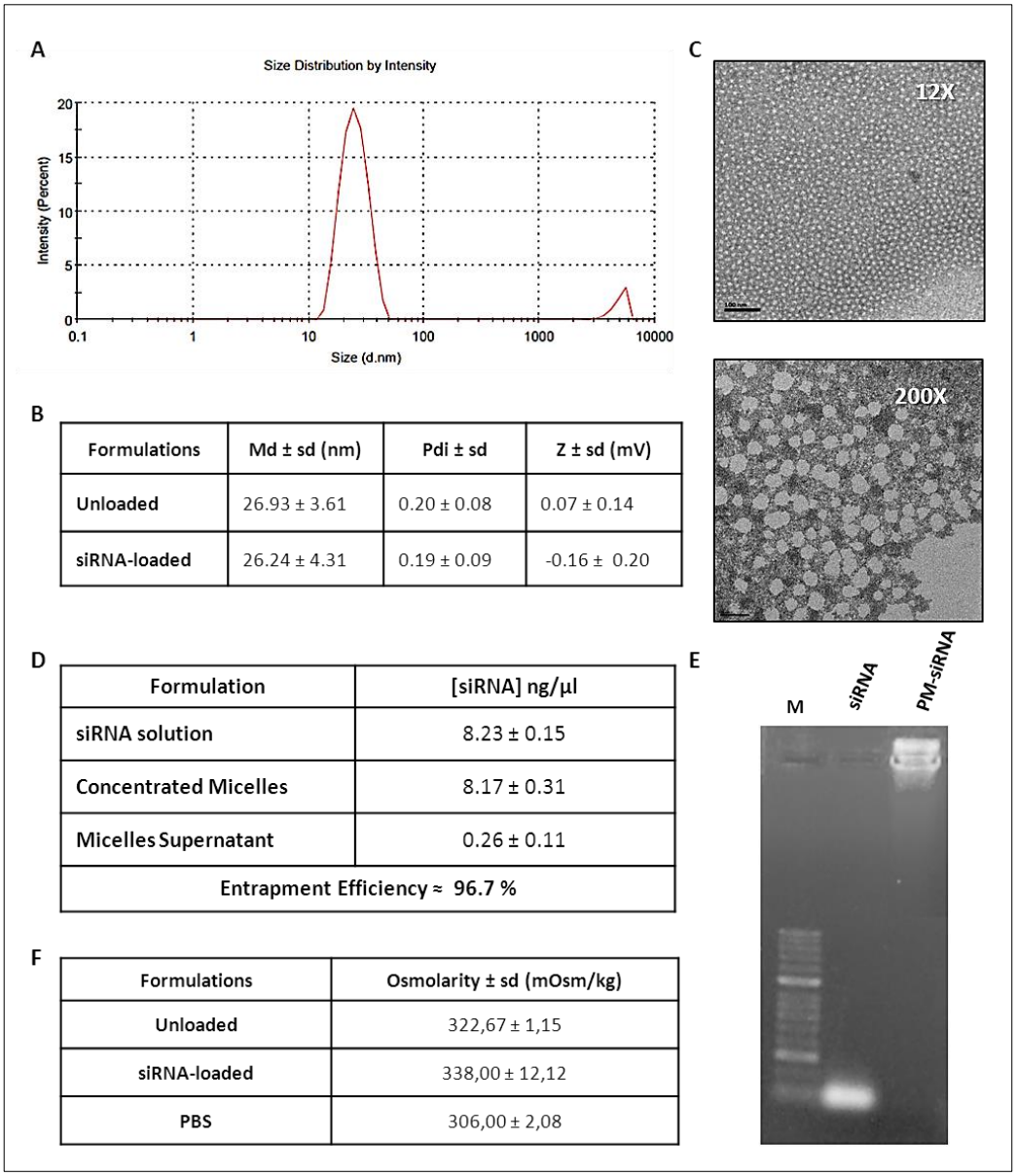
At least three batches of each PM were produced and characterized and the results expressed as the mean±SD. For biological studies, at least 3 replicates, each involving at least two technical replicates were involved in final results expressed as the mean±SD. Statistical analysis was performed in Microsoft Office Excel™ 2007 using the unpaired Student's t-test. Differences were regarded as statistically significant when p-value were smaller than 0.05.

5.3. Results

5.3.1. PM are Technologically Favorable for siRNA Delivery

In order to characterize physicochemically the proposed formulation, several technological tests were performed. The combination of poloxamer F127 and 10K branched PEI-based

polyplexes originated particles with ideal characteristics for siRNA delivery and intravenous administration.



Formulations	Md ± sd (nm)	Pdi ± sd	Z ± sd (mV)
Unloaded	26.93 ± 3.61	0.20 ± 0.08	0.07 ± 0.14
siRNA-loaded	26.24 ± 4.31	0.19 ± 0.09	-0.16 ± 0.20

Formulation	[siRNA] ng/µl
siRNA solution	8.23 ± 0.15
Concentrated Micelles	8.17 ± 0.31
Micelles Supernatant	0.26 ± 0.11
Entrapment Efficiency ≈ 96.7 %	

Formulations	Osmolarity ± sd (mOsm/kg)
Unloaded	322,67 ± 1,15
siRNA-loaded	338,00 ± 12,12
PBS	306,00 ± 2,08

Figure 5. 2. Micelles physicochemical characterization. (A) mean hydrodynamic diameter distribution of PM-siC represented trough the graph of size dispersion by intensity, (B) summary of size, polydispersity and zeta potential, (C) TEM images of PM at different magnifications, (D) siRNA entrapment efficiency determined by a spectrophotometer method, (E) agarose gel retardation assay, and (F) osmolarity values of PM and PBS. Results are expressed as mean±SD (n≥3).

The micelles present a mean diameter around 26 nm, both for loaded and unloaded formulations, and low polydispersity indexes (≤ 0.2). Despite the positively charges associated with PEI, micelles present a neutral charge mainly caused by the presence of PEG at the surface **(Figure 5.2A and B)**. The mean diameter values as well as the low

polydispersity were also confirmed by TEM images, where it is possible to observe micelles of small size and characteristic spherical morphology **(Figure 5.2C).**

In order to confirm if the siRNA was correctly complexed with PEI and associated with micelles, the entrapment efficiency was determined by two distinct methods. The indirect method, that measures the free siRNA concentration at the supernatant after PM centrifugation with filtration, shows entrapment efficiencies higher than 96% **(Figure 5.2D)**. Additionally, no free siRNA fraction was detectable by agarose gel retardation assay in the micelles sample **(Figure 5.2E)**.

Micelles present an osmolarity value slightly superior to the PBS but < 350 mOsm/Kg, which corroborates their adequate applicability for systemic administration **(Figure 5.2F)**.

5.3.2. Complete PM Internalization Occurs After 4 hours of Incubation

The internalization of the fluorescent-labelled micelles was assessed quantitatively by flow cytometry **(Figure 5.3)** and qualitatively by confocal microscopy **(Figure 5.4)**. In order to study the uptake kinetics the cells were incubated with PM at different time-points. With the objective to check if there is any difference in the internalization pattern between CSC and non-CSC cells, MDA-MB-231 and MCF7 ALDH1A1 cells with around 50% of tdTomato+ (CSC) were used **(Figure 5.3C and G)**. It was possible to observe that particles start to be internalized 10 minutes after incubation, but only after 4 hours an internalization of 90% is reached **(Figure 5.3D and H)**. Interestingly, although no significant differences were observed between CSC and non-CSC cells regarding the amount of cells that internalize micelles **(Figure 5.3A and E)**, is possible to observe superior values of fluorescence intensity for CSC cells, which possibly represents a higher number of PM inside each cell **(Figure 5.3B and F)**. Thus, it is possible to predict that tdTomato+ cells internalize significantly more micelles than the tdTomato- ones. This result needs to be further explored in the future to clarify the endocytic pathways and understand the possible different behavior between CSC and non-CSC.

For the qualitative analysis, MDA-MB-231 cells incubated with fluorescent-labeled PM were observed by confocal microscopy. As expected, it is possible to observe micelles internalization as well as co-localization with the cell endosomal vesicles **(Figure 5.4)**.

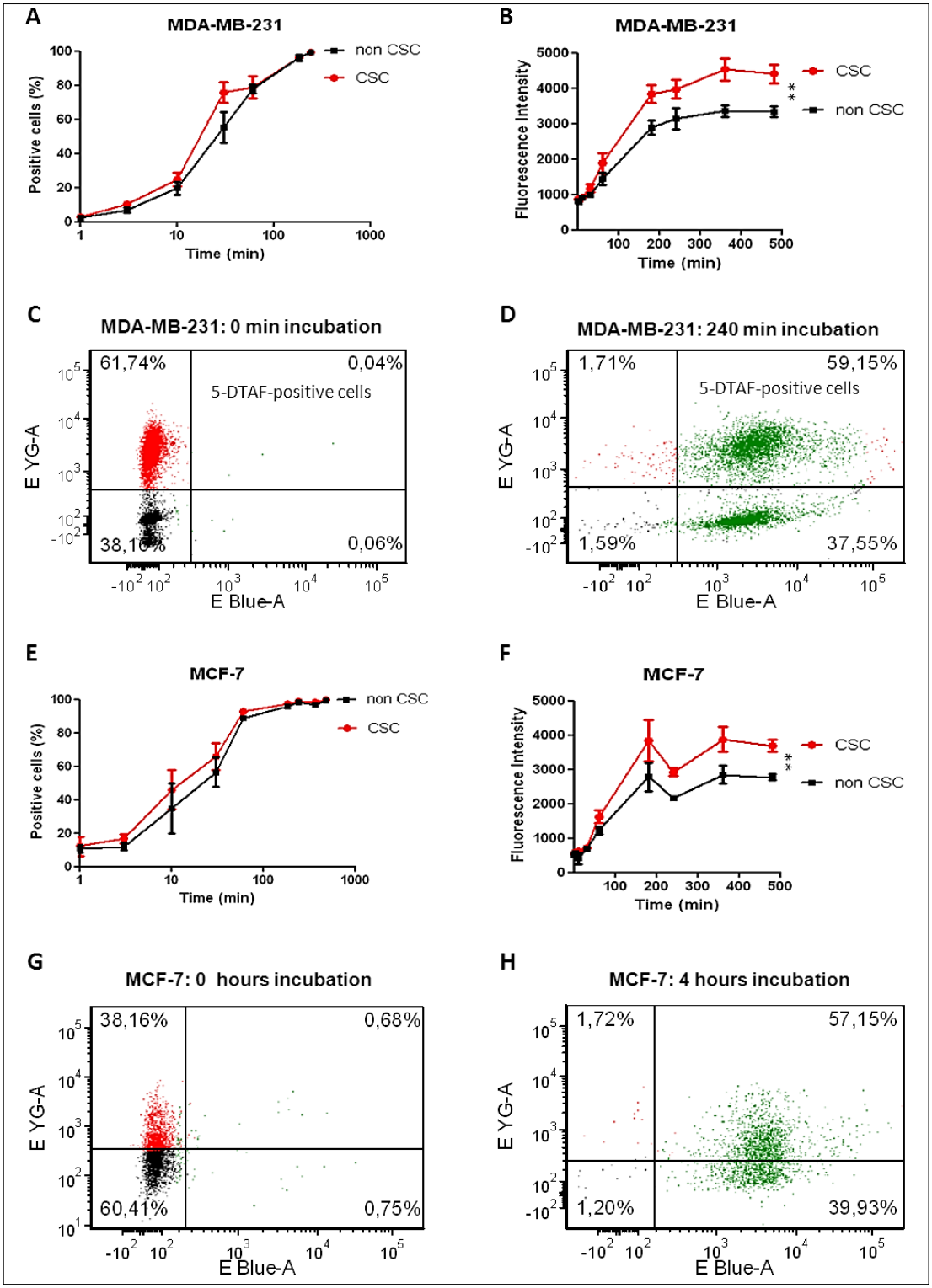


Figure 5. 3. FACS quantification of micelles uptake by MDA-MB-231 and MCF7 ALDH1A1 tdTomato+ versus tdTomato-. The cells were incubated with the 5-DTAF labeled PM and the percentage of cells emitting green fluorescence (Graph A and E) as well as the intensity of fluorescence (Graph B and F) were quantified at different time-points. The bidimensional plots showing the internalization of PM in both subpopulation of CSC and non-CSC at time 0 and time 4 hours are presented in graphs C,D,G and H. Results are expressed as mean±SD (n=3), ** p≤0.01 for de comparison between CSC and non-CSC.

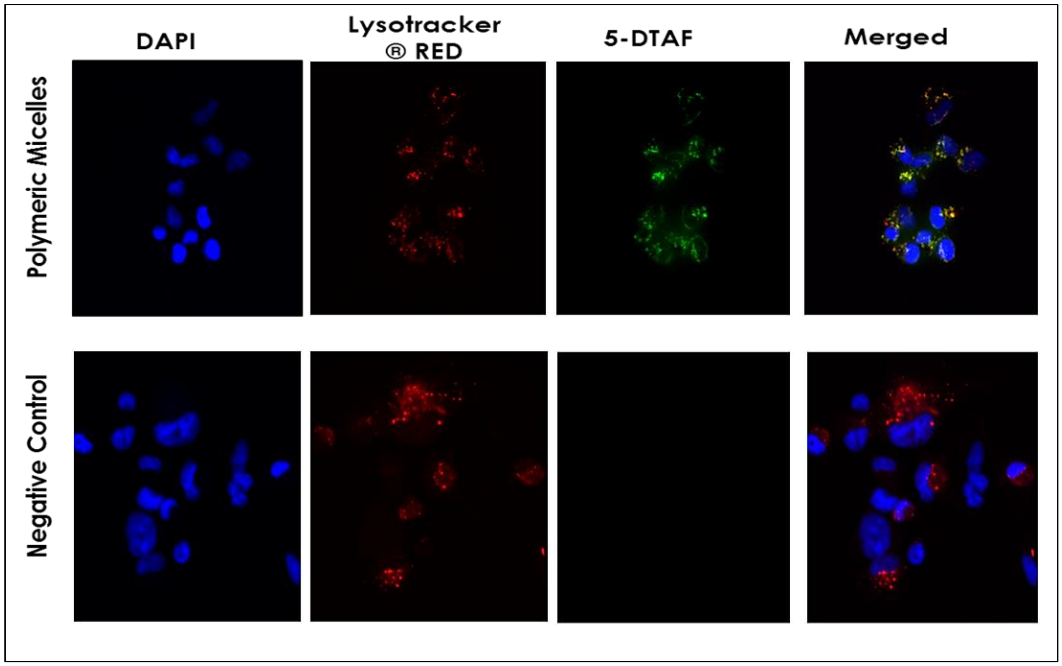
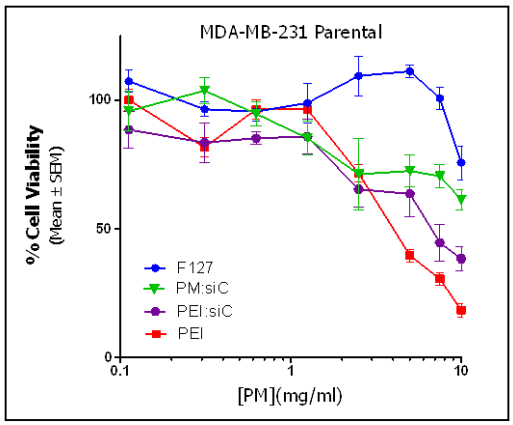
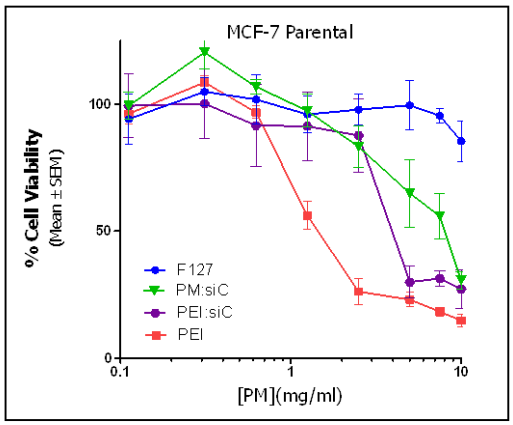


Figure 5. 4. PM internalization visualization throught confocal microscopy. The PM are labeled in green with the 5-DTAF, the endossomal vesicles are labeled in red (Lysotracker® RED and the cells nuclei labeled in blue (DAPI).

A





B

Component	IC50 (mg/ml)
F127	>10
PEI	0,015
PEI:siControl	0,022
PM:siControl	>10

Component	IC50 (mg/ml)
F127	>10
PEI	0,005
PEI:siControl	0,013
PM:siControl	7,26

Figure 5. 5. *In vitro* cytotoxicity of PM and its isolated components. A) Assessment of the effects of PM and isolated components concentration in the cell viability. To facilitate the comparison between samples, the concentration presented for the isolated components is not the real one, but corresponds to the one present in the final PM concentration (for example, in the plot is not being assessed the toxicity corresponding to 5 mg/ml of PEI, but instead the toxicity of the PEI concentration present in 5 mg/ml of PM). B) IC_{50} values for each component and for PM. The values were obtained by interpolation of y=50 from the dose-effect curves fitting using the real concentrations used for each sample. Results are expressed as mean±SD (n=3).

5.3.3. PM do not Present *in vitro* or *in vivo* Toxicity

Effects on cell viability of PM-siRNA as well as its isolated components were assessed *in vitro* via MTT assay in MDA-MB-231 and MCF7 **(Figure 5.5A)** breast cancer cells, with the additional estimation of their IC_{50} values **(Figure 5.5B)**. As observed in both dose-effect graphs and the IC_{50} values, F127 do not cause significant toxicity to the cells. On the other side, PEI alone is highly toxic. However, this PEI-related toxicity appears to be reduced when it is complexed with the negatively charged siRNA and even more reduced in the presence of the F127 shell. Since the concentrations of PM that have demonstrated to be efficient *in vitro* (5 mg/ml) are inferior to their IC_{50} value, is possible to conclude that PM are not expected to significantly cause toxicity after administration.

Before to administrate the PM *in vivo*, a serum stability assay was perform in order to predict the amount of aggregation between the PM and the serum components *in vivo*. **Figure 5.6** represents the mean size distribution curves by intensity for the PM-siRNA incubated with medium-FBS (50%) at the different time-points **(Figure 5.6A)** and for the medium-FBS (50%) **(Figure 5.6B)**.

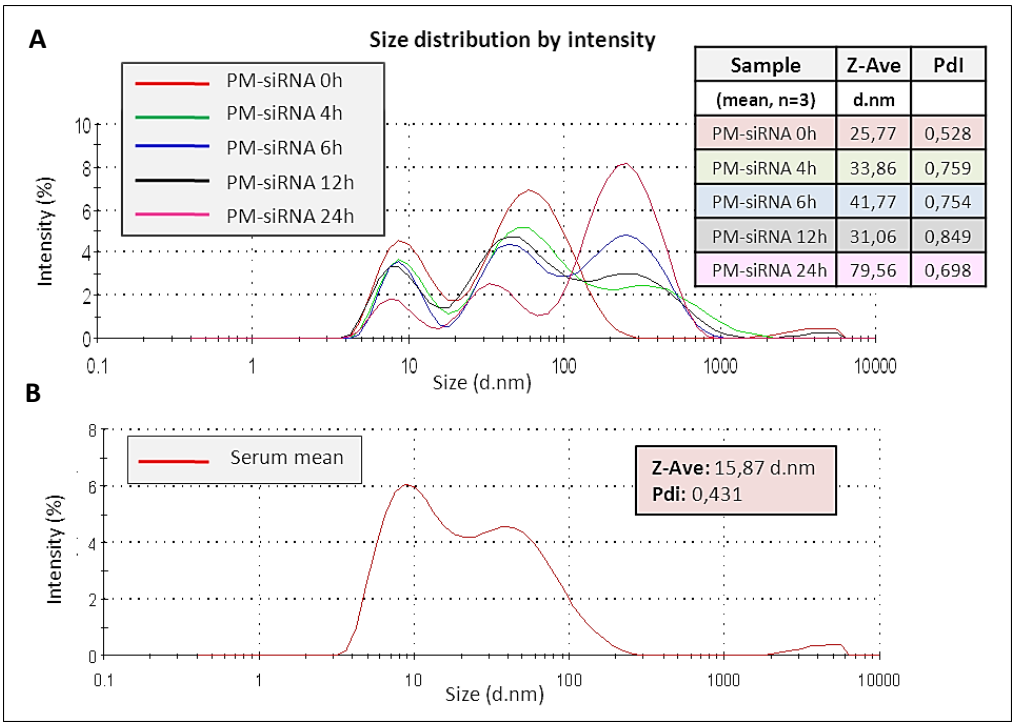
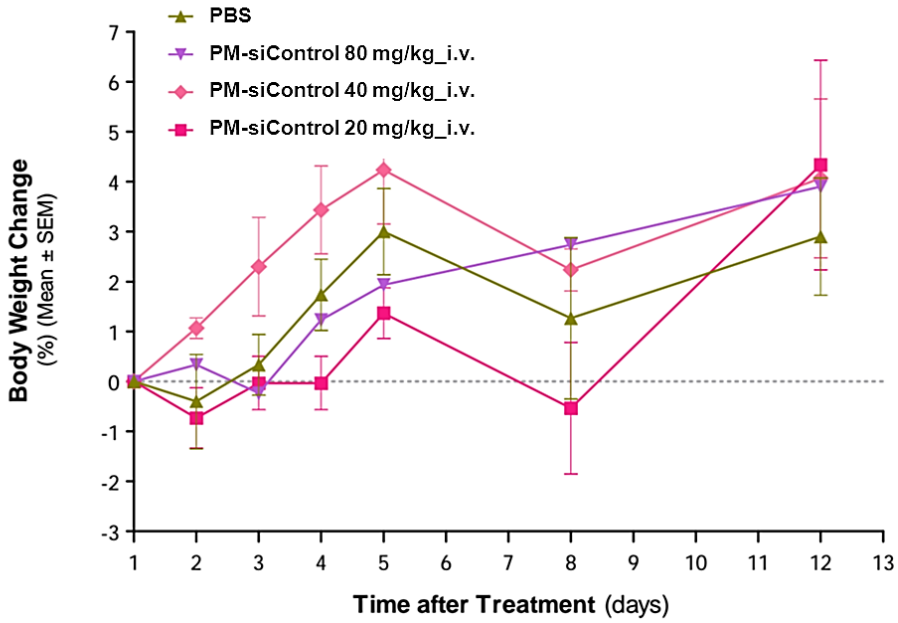


Figure 5. 6. Serum stability assay PM-siRNA. The graphs represent the DLS size distributions by intensity after incubation at different time-points. (A) PMs with siC mean curves at different time-points (0-24 hours). (B) Medium-serum 50% curve. All measurements were performed in triplicate, with three technical samples.

The distribution curves demonstrate some aggregation of the PM with the serum components over the time since it can be observed a reduction of the medium particles fraction (20-100nm) with a consequent increase in the percentage of particles with higher sizes (>100nm). This behavior was especially observed after 12 hours of incubation. Taking into account that we expect that the PM are eliminated before the 12 hours, the verified aggregation do not constitute a major problem for the intravenous *in vivo* administrations of the formulation.



Dose PM-siControl (mg/kg)	Summary Side Effects								
	3 hours post-administration					1 day-12 days post-administration			
	Voluntary movement reduction	Irregular breathing	Motor disturbances	Seizures	Sudden death	Flushed skin	Lacrimation	Dehydration	Hunched position
PBS	0/3 (0%)	0/3 (0%)	0/3 (0%)	0/3 (0%)	0/3 (0%)	0/3 (0%)	0/3 (0%)	0/3 (0%)	0/3 (0%)
80	0/3 (0%)	0/3 (0%)	0/3 (0%)	0/3 (0%)	0/3 (0%)	0/3 (0%)	0/3 (0%)	0/3 (0%)	0/3 (0%)
40	0/3 (0%)	0/3 (0%)	0/3 (0%)	0/3 (0%)	0/3 (0%)	0/3 (0%)	0/3 (0%)	0/3 (0%)	0/3 (0%)
20	0/3 (0%)	0/3 (0%)	0/3 (0%)	0/3 (0%)	0/3 (0%)	0/3 (0%)	0/3 (0%)	0/3 (0%)	0/3 (0%)

Figure 5. 7. PM toxicity *in vivo*. In the graph is presented the body weight variation of animals up to 12 days post-administration of the samples. The table shows the register of adverse side effects monitored during the time of the experiment. Results are expressed as mean±SD (n=3).

The safety of PM was also assessed *in vivo*. For this, PM were administered to healthy mice at increasing concentrations in order to determine the MTD. A maximum administrated dose of 80 mg of PM per kilogram of body weight was well tolerated, without inducing adverse side effects up to 12 days post-administration. As observed in **Figure 5.7**, no significant changes in weight gain neither weight loss were seen in the animals treated with the PM when compared to the control group of animals injected with PBS ($p \leq 0.05$).

5.3.4. PM-siGFP Efficiently Silence the GFP Reporter Gene Expression

PM entrapping a siRNA against GFP were incubated at different times with GFP expressing MDA-MB-231 breast cancer cells. As shown in **Figure 5.8**, a clear and strong qualitative **(Figure 5.8A)** and quantitative **(Figure 5.8B)** reduction of GFP expression were observed 24 hours post-transfection in cells treated with GFP-siRNA when compared with the cells transfected siC.

5.3.5. PM-siAKT2 Reduce the Metastatic Potential of CSC

Next, we examined whether the PM platform could be used to silence a potential therapeutic target related with breast CSC. PM were prepared using the siAKT2 and siC and transfected into MDA-MB-231 and MCF7 breast cancer cells as well as in their CSC fractions isolated as explained in **Chapter 4**. During our study we verified the stemness gene expression after each sorting to guarantee the purity of the samples. The decrease of AKT2 expression in both cell lines was confirmed by qPCR **(Figure 5.9A)**. Subsequently, in order to determine the effects related to AKT2 inhibition in the fraction of CSC, cells invasion and anchorage-independent growth ability were assessed for MDA-MB-231 Td-Tomato+ and MCF7 Td-Tomato+ cells after treatment with the PM-siAKT2 and PM-siC. Regarding the invasive potential of CSC, for both cases the invasiveness of CSC was significantly reduced after PM-siAKT2 transfection **(Figure 5.9B)**. Next, the assessment of the anchorage-independent growth capacity revealed a reduced number of transformed cells for both MDA-MB-231 and MCF7 CSC after treatment with the PM-siAKT2 **(Figure 5.9C)**. Accordingly, it was observed an accentuated impairment of colonies formation after the treatment with PM-siAKT2 **(Figure 5.9D).**

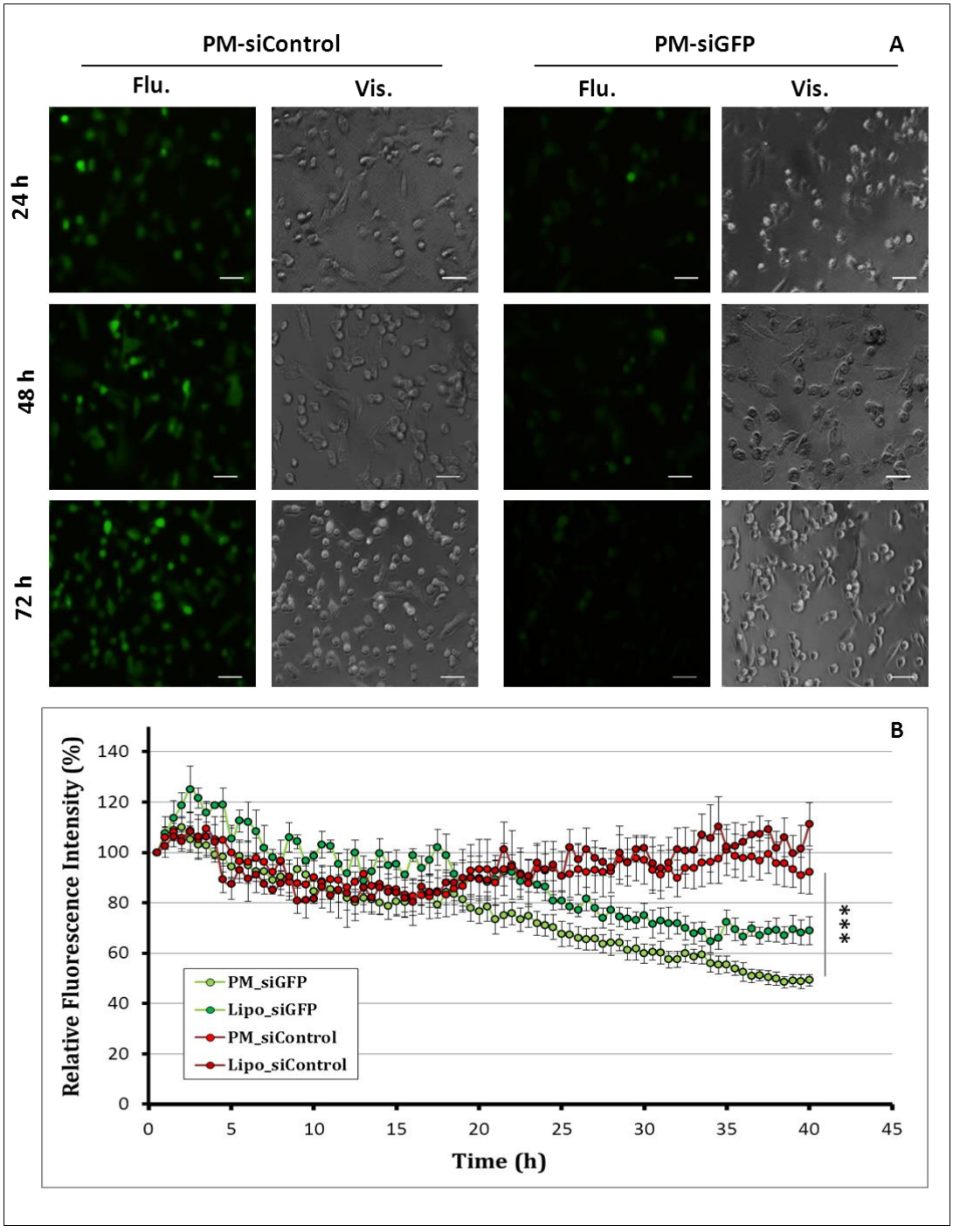


Figure 5. 8. GFP reporter assay for PM-siGFP biological efficacy assessment. (A) Fluorescent images of MDA-MB-231/GFP cells were taken 24, 48 and 72 hours after incubation with PM-siGFP and PM-siC. (B) Time-lapse experiment in which fluorescent images of cells incubated with siGFP and siC associated with PM or Lipofectamine® 2000 were captured at each 30 minutes during 72 hours. The values represent the fluorescent intensity detected for each time-point. Results are expressed as mean±SD (n=3), *** p≤ 0.001 for the comparison between PM-siGFP and PM-siC.

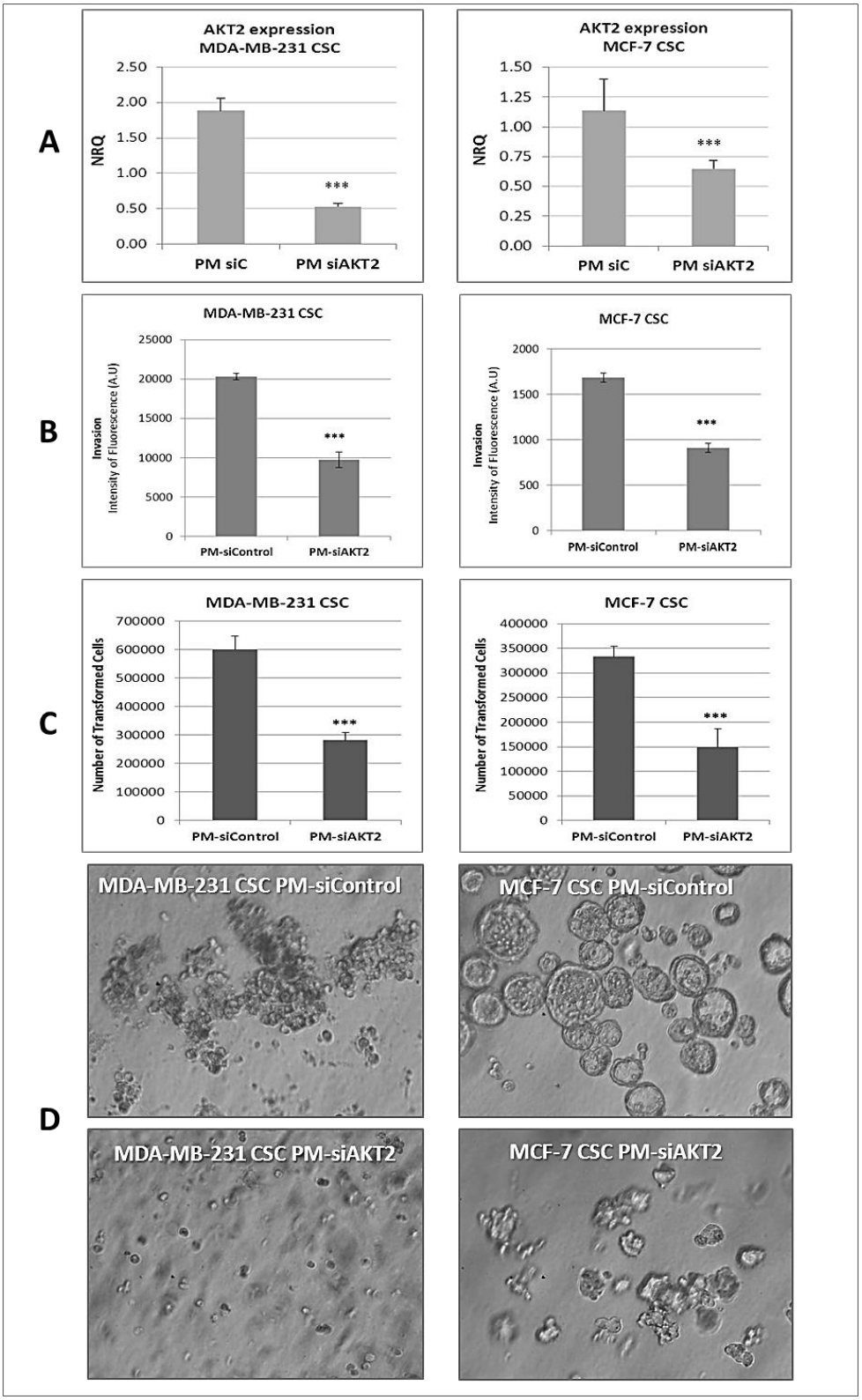


Figure 5. 9. PM-siAKT2 effects in MDA-MB-231 and MCF7 cells. (A) The reduction of AKT2 gene expression was detected in both cell lines through qPCR. The presented values are normalized to the housekeeping genes (Actin and GADPH). (B) Quantification of the number of invasive cells after incubation with PM-siAKT2 versus PM-siC. (C) Quantification of the number of transformed cells after incubation with PM-siAKT2 versus PM-siC. (D) Colonies formation on the soft agar after incubation of cells with PM-siAKT2 versus PM-siC. Results are expressed as mean±SD (n=3), *** $p \leq 0.001$ for the comparison between PM-siC and PM-siAKT2.

5.4. Discussion

In this work PM were chosen as siRNA delivery systems due to their well-known versatility to produce, among others, systems presenting long circulating profiles in the bloodstream and able to improve the cellular uptake of compounds, increasing its therapeutic efficacy (3, 4). Among the amphiphilic polymers used to produce micelles, poloxamers enjoy special prominence due to their biocompatible and biodegradable nature, well-established status as drug delivery and biomedical excipients, capacity to improve the transfection of genetic material, and to support endosomal release of endocytosed nanocarriers (2, 5, 6). Regarding the choice of the amphiphilic polymer, the Pluronic® F127 presented adequate physical properties regarding size and Pdi as referred in **Chapter 3**. Similar physicochemical results were obtained with PM of poloxamers encapsulating insulin (1). Since poloxamers present neutral charge at the physiological pH, it was necessary to introduce a cationic component to formulation in order to obtain required positive charge to complex the anionic siRNA, so the branched PEI 10K was chosen as the polycation to produce the polyplexes (as explained in **Chapter 3**). Like this we can enjoy from the benefits of both PEI and poloxamers regarding transfection efficiency, and PEGylated surface of PM.

The morphological analysis has shown micelles with spherical shape and md close to 27 nm **(Figure 5.2)**, corroborating the results of DLS. The lower size of micelles composed by F127 is explained by its lower critical micelle concentration (CMC) and hydrophilic-lipophilic balance (HLB) values, resulting in more compact micelles (1).

Due to their small size and the enhanced transfection efficiency capacity of both components, PM are quickly and efficiently internalized by cells **(Figure 5.3)** and efficiently promote the silencing of the gene of interest **(Figure 5.8 and 5.9)**. Moreover, the cytotoxicity assay reveals that the presence of F127 reduce the PEI-related toxicity, probably due to the reduction of its surface cationic groups. Therefore, the system do not present significant toxicity both *in vitro* **(Figure 5.5)** as well as *in vivo* **(Figure 5.7)**, which is related to the biocompatibility of poloxamers explaining their use on a variety of biomedical applications including drug delivery (7, 8).

Another advantage about the presence of poloxamers in the formulation was evidenced by the serum stability assays. The results showed that there is no aggregation with serum components up to 12 hours of incubation, suggesting that this PM may be adequate for intravenous administration *in vivo* **(Figure 5.6)**.

Other research groups developed micellar systems for siRNA delivery proving the utility of this type of systems. For example, Liu L, *et al.* (2016) synthesized an amphiphilic and

biodegradable ternary copolymer conjugated with folate as ligand, polyethylenimine-graft-polycaprolactone-block-poly(ethylene glycol)-folate (PEI-PCL-PEG-Fol) for targeted siRNA delivery via folate-FR recognition (9). They obtained stable micelles with hydrodynamic diameters about 100 nm and a zeta potential of +8.6 mV. The presence of folate was responsible for the increased cellular uptake and *in vitro* gene knockdown observed in a folate receptor enriched cell line (SKOV-3 cells). Moreover, these PEI-PCL-PEG-Fol/siRNA micelles exhibited high stability *in vivo* and a long circulation half-life, which allowed 17% of the intravenously injected siRNA to reach the tumor mainly due to their effective folate targeting (10). Furthermore, the study of Salzano G, *et al*. (2015) brings the interesting possibility of micelles multifunctionalization (10). In their study they developed self-assembly PM able to efficiently co-load an anti-survivin siRNA and a chemotherapeutic agent, such as PTX (survivin siRNA/PTX PM) and evaluated their applicability *in vivo* in an animal model of PTX-resistant ovarian cancer. They obtained a significant downregulation of survivin expression in tumor tissues together with a potent anticancer activity of survivin-siRNA/PTX PM, while the tumors remained unaffected with the same quantity of free PTX. These results underline the importance of the combined therapy in the treatment of chemoresistant tumors (11).

The versatility of the poloxamers used in our system opens the possibility of creating functionalized systems by conjugation of moieties to the PEG surface, and the co-encapsulation of polyplexes and anticancer drugs, benefiting from the advantages of the combined therapy. In fact, poloxamers have shown to reduce the MDR capacity of cancer cells through a selective energy depletion and inhibition of the drug efflux systems, improving especially the therapeutic efficacy of the drugs in multidrug resistant tumors (12-14). Exploring these characteristics of poloxamers, a formulation of PM encapsulating Doxorubicin (SP1049C) granted orphan designation by FDA to treat advanced carcinoma of the esophagus and advanced gastric cancers, proving its value in the development of systems for cancer treatment (15, 16). Additionally, poloxamers present cryoprotective properties, allowing the lyophilization of the system to obtain a powder with good redispersion profile, allowing the increase of the product stability and shelf-life, and reducing the costs associated to liquid formulations (1).

Due to their unique phenotype, CSC are resistant to conventional therapies (17, 18). Therefore, it is of major importance to find an adequate target against CSC and an effective delivery system able to reach them, otherwise the reservoir of resistant CSC will cause recurrence of aggressive tumor and metastatic growth over the time (19, 20). We used a CSC model based on the tdTomato expression under the control of CSC specific ALDH1A1 promoter, in order to study the efficacy of AKT2 silencing in CSC fraction. This CSC model

was previously extensively validated *in vitro* and *in vivo* (21). In **Chapter 4**, we have demonstrated that downregulating AKT2 using a siAKT2 have an important role in the EMT impairment and the consequent reduction metastatization and malignancy of cancer cells with special importance in CSC. After treatment with the PM-siAKT2, the transformation capacity of CSC fractions (MDA-MB-231 and MCF7) was significantly abolished ($p \leq 0.001$). Similarly, the invasion capacity of CSC significantly decreased ($p \leq 0.001$) after the transfection with the PM-siAKT2 **(Figure 5.9)**. These results suggest that AKT2 silencing strategy may be successful as a target for future RNAi-based antitumor therapies particularly on the challenging fraction of highly aggressive CSC.

The internalization studies demonstrated that micelles are able to be taken up by cells through endocytosis, being possible to observe their co-localization with the endosomal vesicles **(Figure 5.4)**. This result is in agreement with the biological efficacy studies performed, since the internalization by endocytosis of polyplexes/micelles containing the siRNA is the first step to become available to perform is biological function inside the cell. It also revealed a significant preferential uptake of PM by CSC ($p \leq 0.01$) **(Figure 5.3)**, resulting in a high biological therapeutic efficacy **(Figure 5.9)**. The mechanism involved in the enhancement of transfection efficiency by poloxamers is the same underlying its effects as MDR chemosensitizers. By interfering with the cellular membrane, poloxamers inhibit the ATPase activity and the efflux function of P-gp, improving the efficacy of anticancer drugs (14, 22). Moreover, poloxamers interfere with mitochondrial function showing to enhance proapoptotic signaling and decrease tumorigenicity of cancer cells by altering the epigenetic regulation of cells mainly in MDR over non-MDR cells (12, 15, 23). Taking into account that CSC display some of the characteristics of MDR cells, like overexpression of efflux systems, poloxamers present the capacity to enhance the anticancer activity of drugs (e.g. Doxorubicin), increase the transfection efficiency of OGN, and to decrease the tumorigenicity and aggressiveness of tumor cells, especially in CSC sub-population (15).

5.5. Conclusions

In this study, an innovative nanocarrier system composed by Pluronic® F127 micelles associated with PEI-based polyplexes has been developed with the objective to overcome the major drawbacks usually associated to the genetic material delivery systems, namely siRNA degradation, insufficient transfection efficiency or excessive nanoparticles-related toxicity.

The proposed system seems to gather the requirements for an efficient and safe transport of siRNA in terms of their physicochemical characteristics, internalization capacity,

biological efficacy and toxicity profile. Moreover we also observed a reduction of the invasion and transformation capacities of breast cancer cells, when transfected with PM entrapping the polyplexes with the siRNA against the gene of interest, with significant positive results observed mainly in the so feared CSC population.

These results make us believe that this new formulation will serve as a technological platform for the development of systems to encapsulate different siRNAs, as well as other kind of genetic material, and bring new insights into the field of cancer gene therapy.

The next step in the development and optimization of these PM would be the introduction of specific targeting moieties covalently linked to the polymers chains and assess to which extent the active targeting could even improve the biological and therapeutic efficacy of the system. It would be also priority to increase the loading capacity of the micelles to undergo *in vivo* assays. First it should be performed a biodistribution study to track how PM behave once in the blood circulation. Afterwards an *in vivo* efficacy study in a breast and/or colon cancer mouse model using the PM carrying the siAKT2 versus siC will be also performed.

Another future objective of this work is the creation of a multifunctional system where can be combined the delivery of both conventional drugs and genes. The system developed presents adequate properties to deliver both hydrophobic and hydrophilic substances, added at different steps of the production method. Therefore, the design of a nanosystem combining an hydrophobic drug, such as the PTX or zileuton, and the hydrophilic polyplexes of a certain siRNA, chosen depending on the genetic pattern of the disease, could be a encouraging strategy in the future of the combined and personalized therapy.

5.6. References

1. Andrade F, Fonte P, Oliva M, Videira M, Ferreira D, Sarmento B. Solid state formulations composed by amphiphilic polymers for delivery of proteins: characterization and stability. International journal of pharmaceutics. 2015;486(1-2):195-206.

2. Kabanov A, Zhu J, Alakhov V. Pluronic block copolymers for gene delivery. Advances in genetics. 2005;53:231-61.

3. Andrade F, Videira M, Ferreira D, Sarmento B. Micelle-based systems for pulmonary drug delivery and targeting. Drug Delivery Letters. 2011;1(2):171-85.

4. Andrade F, Rafael D, Videira M, Ferreira D, Sosnik A, Sarmento B. Nanotechnology and pulmonary delivery to overcome resistance in infectious diseases. Adv Drug Deliv Rev. 2013;65(13-14):1816-27.

5. Chen J, Luo J, Zhao Y, Pu L, Lu X, Gao R, et al. Increase in transgene expression by pluronic L64-mediated endosomal/lysosomal escape through its membrane-disturbing action. ACS applied materials & interfaces. 2015;7(13):7282-93.

6. Yang Z, Sahay G, Sriadibhatla S, Kabanov AV. Amphiphilic block copolymers enhance cellular uptake and nuclear entry of polyplex-delivered DNA. Bioconjug Chem. 2008;19(10):1987-94.

7. Jung HH, Park K, Han DK. Preparation of TGF-beta1-conjugated biodegradable pluronic F127 hydrogel and its application with adipose-derived stem cells. Journal of controlled release : official journal of the Controlled Release Society. 2010;147(1):84-91.

8. Diniz IM, Chen C, Xu X, Ansari S, Zadeh HH, Marques MM, et al. Pluronic F-127 hydrogel as a promising scaffold for encapsulation of dental-derived mesenchymal stem cells. Journal of materials science Materials in medicine. 2015;26(3):153.

9. Batrakova EV, Li S, Alakhov VY, Miller DW, Kabanov AV. Optimal structure requirements for pluronic block copolymers in modifying P-glycoprotein drug efflux transporter activity in bovine brain microvessel endothelial cells. The Journal of pharmacology and experimental therapeutics. 2003;304(2):845-54.

10. Liu L, Zheng M, Librizzi D, Renette T, Merkel OM, Kissel T. Efficient and Tumor Targeted siRNA Delivery by Polyethylenimine-graft-polycaprolactone-block-poly(ethylene glycol)-folate (PEI-PCL-PEG-Fol). Molecular pharmaceutics. 2016;13(1):134-43.

11. Salzano G, Navarro G, Trivedi MS, De Rosa G, Torchilin VP. Multifunctional Polymeric Micelles Co-loaded with Anti-Survivin siRNA and Paclitaxel Overcome Drug Resistance in an Animal Model of Ovarian Cancer. Molecular cancer therapeutics. 2015;14(4):1075-84.

12. Alakhova DY, Rapoport NY, Batrakova EV, Timoshin AA, Li S, Nicholls D, et al. Differential metabolic responses to pluronic in MDR and non-MDR cells: a novel pathway for chemosensitization of drug resistant cancers. Journal of controlled release : official journal of the Controlled Release Society. 2010;142(1):89-100.

13. Batrakova EV, Kelly DL, Li S, Li Y, Yang Z, Xiao L, et al. Alteration of genomic responses to doxorubicin and prevention of MDR in breast cancer cells by a polymer excipient: pluronic P85. Mol Pharm. 2006;3(2):113-23.

14. Batrakova EV, Li S, Elmquist WF, Miller DW, Alakhov VY, Kabanov AV. Mechanism of sensitization of MDR cancer cells by Pluronic block copolymers: Selective energy depletion. British journal of cancer. 2001;85(12):1987-97.

15. Alakhova DY, Zhao Y, Li S, Kabanov AV. Effect of doxorubicin/pluronic SP1049C on tumorigenicity, aggressiveness, DNA methylation and stem cell markers in murine leukemia. PloS one. 2013;8(8):e72238.

16. Danson S, Ferry D, Alakhov V, Margison J, Kerr D, Jowle D, et al. Phase I dose escalation and pharmacokinetic study of pluronic polymer-bound doxorubicin (SP1049C) in patients with advanced cancer. Br J Cancer. 2004;90(11):2085-91.

17. Yu Y, Ramena G, Elble RC. The role of cancer stem cells in relapse of solid tumors. Front Biosci (Elite Ed). 2012;4:1528-41.

18. Dragu DL, Necula LG, Bleotu C, Diaconu CC, Chivu-Economescu M. Therapies targeting cancer stem cells: Current trends and future challenges. World J Stem Cells. 2015;7(9):1185-201.

19. Nandy SB, Gangwani L, Nahleh Z, Subramani R, Arumugam A, de la Rosa JM, et al. Recurrence and metastasis of breast cancer is influenced by ovarian hormone's effect on breast cancer stem cells. Future Oncol. 2015;11(6):983-95.

20. Sehl ME, Shimada M, Landeros A, Lange K, Wicha MS. Modeling of Cancer Stem Cell State Transitions Predicts Therapeutic Response. PLoS One. 2015;10(9):e0135797.

21. Gener P, Gouveia LP, Sabat GR, de Sousa Rafael DF, Fort NB, Arranja A, et al. Fluorescent CSC models evidence that targeted nanomedicines improve treatment sensitivity of breast and colon cancer stem cells. Nanomedicine. 2015;11(8):1883-92.

22. Batrakova EV, Li S, Alakhov VY, Elmquist WF, Miller DW, Kabanov AV. Sensitization of cells overexpressing multidrug-resistant proteins by pluronic P85. Pharmaceutical research. 2003;20(10):1581-90.

23. Minko T, Batrakova EV, Li S, Li Y, Pakunlu RI, Alakhov VY, et al. Pluronic block copolymers alter apoptotic signal transduction of doxorubicin in drug-resistant cancer cells. Journal of controlled release : official journal of the Controlled Release Society. 2005;105(3):269-78.

CHAPTER 6

General Conclusions and Future Perspectives

Cancer is a higly prevalent disease having a major impact in the population health worldwide. Even though current treatments have improved the overall survival and life quality of patients, and despite the new generation of targeted anticancer drugs, cancer remains difficult to treat and control. Consequentely, the development of therapy-resistant metastasis that cause cancer relapse is still frequent. Despite the advancements observed in the fields of molecular biology and biotechnology allowing a better understanding of the mechanism of carcinogenesis and disease progression, as well as the development of improved therapies, advanced cancer is generally an incurable disease. As referred, the conventional treatments have improved the survival and cure rate of patients; however there are some associated drawbacks, mostly concerning high toxicity and serious side-effects, which may even cause death. More than half of the treated patients suffer from disease relapse, including the presence of distant metastasis in other organs. The rapid emergence of therapy resistance seen in the aggressive, metastatic forms of the disease is, by far, the biggest obstacle for the success of current cytotoxic and cytostatic therapies. Indeed, many of the newly developed cancer drugs elicit powerful initial responses, leading to dramatic effects on tumor reduction and disease control, but still modest results regarding of long-term overall survival (1-3).

Cancer maintenance, resistance to therapy and metastatic disease seem to be sustained by the presence of CSC within the tumors. These cells retain the capacity of repopulating the tumor, while being insensitive to conventional anticancer therapies, antimitotic agents or radiation. It is still not clear if the CSC are originated from epithelial stem cells (the ones essential to maintain proliferative homeostasis) or result from accumulation of mutations from differentiated cells. In this scenario, the new cancer therapies under development should fulfill two conditions: (i) reduce the toxicity displayed in current therapies and most importantly, (ii) effectively target and eliminate CSC to overcome drug resistance and tumor recurrence (4, 5).

One major difference between conventional chemotherapies and targeted therapies with respect to the development of resistance is that resistance to targeted therapies follows a more predictable path, being therefore, easier to identify the resistance mechanism and to choose the more appropriate treatment. However, it remains a major challenge to predict which patients will develop metastatic disease. As a result, describing specific targets or signaling pathways is one of the pillars of current cancer research, although most tumors are still treated with conventional cytotoxic therapies. Thus, the development of more personalized and targeted anticancer therapies is essential. In that context the importance of gene therapy gained a new breath in the last decades. This is a result of the important advances observed in the field of molecular biology and particle engineering technology,

which allowed, respectively the characterization of new therapeutic targets and the development of new and improved formulations (4).

Recent advances in genomics make possible now to consider the hypothesis that each tumor has an independent genetic identity and to enumerate all of the genetic lesions in specific cancers. These approaches will yield critical information regarding the biological alterations signature in different tumors and are increasingly being applied prospectively to patient samples in the clinic. At the same time, complementary approach to decipher the molecular basis of malignant transformation depends upon the application of genome scale tools to annotate the function of genes involved in cancer initiation and progression. Over the past several years, have been developed genome scale RNAi, CRISPR and open reading frame expression libraries that permit a systematic evaluation of genes involved in cancer initiation and maintenance. Using these libraries, it was possible to performed screens in a panel of human cancer cell lines to systematically identify cancer vulnerabilities. By combining these functional approaches with information derived from mapping the structural abnormalities present in cancer genomes, have been identified several new oncogenes that contribute to cancer development. The ability to systematically manipulate gene expression at genome scale now provides opportunities to investigate the function of genes involved in cancer initiation and maintenance, which will provide a framework for therapeutic strategies. For example, assays that apply massive parallel sequencing approaches to evaluate tumor tissue or blood from clinical samples for evidence of genomic harbingers of therapy response and resistance are becoming increasingly used. There are various types of approaches that can be used, including active monitoring of patients either during or at the end of their therapeutic regimen by comparison to a baseline sample. The sensitive and quantitative nature of these assays can pinpoint patients with therapy resistance, by virtue of unaltered prevalence of mutations relative to the baseline, or also identify patients with an incomplete response such as minimal residual disease (MRD). Before the treatment, the analysis of the disease genotype could be useful for the selection of the potential targeted therapy. Therapeutic response monitoring using biomarkers is also possible during the course of treatment and can indicate those non-responding patients that might benefit from another therapy. Finally, post-mortem genomic analyses of therapy resistant disease can be obtained from banked metastatic lesions to inform about the genomic nature of therapy resistance. These studies can be incredibly informative towards future monitoring paradigms, therapeutic combinations or new drug design efforts to fight acquired resistance (6-8). Because of that, worldwide research groups have been actively focused in the development of molecular therapies that target specific oncoproteins, with the purpose of developing personalized therapies (e.g. against EGFR,

HER2, FGFR, BRAF, MEK, PI3K, AKT, mTOR or IGF1-R) for patients displaying specific genetic lesions or pathways deregulations. One of the main objectives nowadays and area of intensive research is to identify new predictive biomarkers of response to diverse treatments as well as biomarkers of primary resistance (*de novo*) and secondary treatment (9, 10).

Still in the context of advance biomarker and drug target discovery, there are also groups that are focused on the proteomic analysis of the cancer secretome and in the characterization of the mechanisms adopted by cancer cells to communicate amongst themselves as well as with their microenvironment during tumorigenesis. Most secreted proteins contain a signal peptide that directs their sorting to the extracellular space through the endoplasmic reticulum–Golgi secretory pathway. One of the most striking observations when secretome profiles are carefully analyzed is that they contain hundreds of theoretical intracellular proteins. Recent reports showing intracellular proteins with alternative extracellular functions, metastasis localizations could be relevant in tumorigenesis and could lead to novel therapies against cancer. Taking into consideration that metastasis depends on a gene program expressed by the tumor microenvironment, for example with the secretion of prosurvival factors by stromal cells during the colonization phase of metastasis, blockage of this crosstalk between tumor cells and their microenvironment through the use of chemical inhibitors could be very promising in prevent metastasis formation. This dependency can also be exploited to improve diagnosis and treatment of patients with advanced cancer (11, 12).

In this work we focused in the characterization of the pathway related with the AKT2 oncogene, since AKT2 is one of the three AKT isoforms that have shown to be amplified in solid tumors. This oncogene is increased in response to apoptosis and its activation is also associated with PI3-K related effects and TWIST-promoted metastatic process by enhancing cell migration and invasion (13, 14). We have shown that the silencing of AKT2 signaling pathway both in bulk and in sub-populations of CSC is able to block their tumorigenic potential, and reverting them to a more epithelial and noninvasive phenotype, as demonstrated by the reduction in cell migration, proliferation, invasion and transformation capabilities after the treatment with the siAKT2. We also demonstrate that the silencing of AKT2 can act differently accordingly the cells phenotype. In cells that present TWIST (a major downstream effector of AKT2) we were able to detect the EMT reversion through the restoration of E-cadherin expression and the decrease of mesenchymal markers (Vimentin, N-cadherin and Fibronectin) expression. For cells that do not express TWIST, the silencing of AKT2 reduced the mTOR and increase the GSKβ expression, contributing for the reduction of cells tumorigenic potential. Also, in these cell lines the AKT2 silencing promotes

alteration in apoptosis markers like p53 and Bcl-2. The novelty of this study relies on the evidence that AKT2 silencing is also effective in the isolated subpopulation of CSC, both in breast and colon cancer, since we truly believe that the specific targeting of this cell sub-population will improve the clinical outcomes by reducing the cancer resistance and recurrence.

In this work we propose the silence of AKT2 as a target using an RNAi approach, with the final goal of developing a novel gene therapy-based system. However another strategy that could be worthy to explore in the future is the chemical inhibition of AKT2. CCT128930 is a novel, selective, and potent AKT2 inhibitor that blocks AKT2 activity exhibiting 28-fold greater selectivity for AKT2 than for the other isoforms. CCT128930 exhibited marked anti-tumor responses and inhibited the phosphorylation of a range of downstream AKT2 biomarkers in multiple tumor cell lines both *in vitro* and *in vivo*, arising as a new anticancer drug (15). Moreover, the combination of AKT2 silencing with a traditional chemotherapeutic agent, such as Doxorubicin or PTX, can represent an important future therapeutic approach forward the CSC eradication and improved anticancer therapy.

The main goal of the present study is to develop a therapeutic system that reduce the cancer therapy-related secondary effects, and specifically target the subpopulation of CSC within the tumors to overcome resistance to therapy and recurrent metastatic and advanced disease. Both criteria could be accomplished using innovative targeted nanomedicines for drug and/or gene delivery, which is widely expected to create innovative therapeutics in cancer therapy thanks to the selective delivery of higher concentrations of encapsulated drugs, with reduced systemic toxicity, to tumor lesions trough the well-known EPR effect. Based on these characteristics, the first generation nanomedicines already in clinical practice or under clinical evaluation have been presenting improved therapeutic efficacy than the conventional drug formulations, and have changed the scenario of oncological diseases therapy (16).

Of note, ubiquitously targeting cells within a tumor is not always feasible because some drugs cannot diffuse efficiently and the random nature of the passive targeting (EPR effect) makes difficult to control the process. This lack of control may also be responsible for inducing the problematic MDR. Thus, one strategy, to further enhance targeting of drugs to cancer cells including CSC, is the conjugation of nanocarriers with molecules that bind to overexpressed antigens or receptors on the target cells. These second generation vectors are especially useful in tumors that do not exhibit EPR effect or cases where the permeability of the vessels is not compromised (i.e. small metastasis). An additional advantage for using targeting moieties for drug delivery into cancer cells is that drugs will

benefit from entering inside tumor cells by receptor-mediated endocytosis, providing higher concentration levels of active drug compounds within the tumor cell (17).

The nanodelivery system used in this work consisted in PEI-based polyplexes associated to PM composed by poloxamers prepared by thin FH technique to deliver siAKT2. PEI was used due to its well-recognized capacity to complex and transfect genetic material into cells. PM were chosen mainly due to its easy production, small size, versatility to encapsulate both hydrophobic and hydrophilic compounds, and the observed capacity of poloxamers to modify the biological response and decrease the MDR of many cancer cells. Moreover, the presence of PEG provides the formulation with stealth properties, allows the particles modification with several targeting ligands, and facilitates the lyophilization of the system. Regarding the last one, the inherent cryoprotectant properties of PEG reduce aggregation of particles during lyophilization without the need of additional cryoprotectants, which simplifies the formulation and could impact in their stability and safety profile. This question is important in technological terms since a future step of development of the presented system relies in the production of powders by lyophilization in order to increase its long-term storage stability (18-20).

After production and physicochemical characterization, biological assessment of formulations was performed in several cancer cell lines. No significant signs of cytotoxicity were observed as determined by MTT (*in vitro*) and MTD (*in vivo*), thus, no substantial toxicity is expected at therapeutic doses. The presence of F127 in the formulation not only serves to increase the particles functionality and their transfection and biological efficiency, but also proved to significantly reduce the PEI-associated toxicity. Furthermore, PM have shown to be preferentially taken up by the CSC sub-population, thus suffering a sort of passive targeting to CSC, which could be predictive indicator of its therapeutic efficacy.

In conclusion, amphiphilic polymers-based formulations combined with PEI presented appropriate characteristics for delivery of siRNAs and achieve a strong gene silencing, including in CSC. These formulations serve as a platform for future development of improved gene delivery systems.

The ultimate goal of this study is to develop a multifunctional targeted nanomedicine against breast and colorectal CSC by: i) using a specific targeting for CSC surface receptors (i.e. CD44 for breast and CD133 or CXCR4 for colon) and ii) combine classical chemotherapy with a specific siRNA against CSC. Thus, the next steps regarding the improvement of this system will be the addition of a chemotherapeutic agent to the formulation and the conjugation with targeting moieties **(Figure 6.1)**. By efficiently targeting and killing CSC is expected to significantly circumvent CSC-related resistance, reduce the number of metastasis and tumor recurrence, and increase patient overall survival.

After perform *in vitro* and *in vivo* preclinical validation of our nanoconjugate targeting CSC, the final step would be to initiate scale-up and regulatory studies.

Following the evolution that has been observed in this field during the last decades, is expected in the near future a further increase in the development of optimized formulations based on nanoparticles for gene delivery and/or to improve the properties of several chemotherapeutic drugs. Due to the well-known advantages presented by these systems would not be surprising to see an exponential increase of marketing authorization and commercialization of multifunctional nanotechnology products, which we hope will bring a new breath in the battle against cancer.

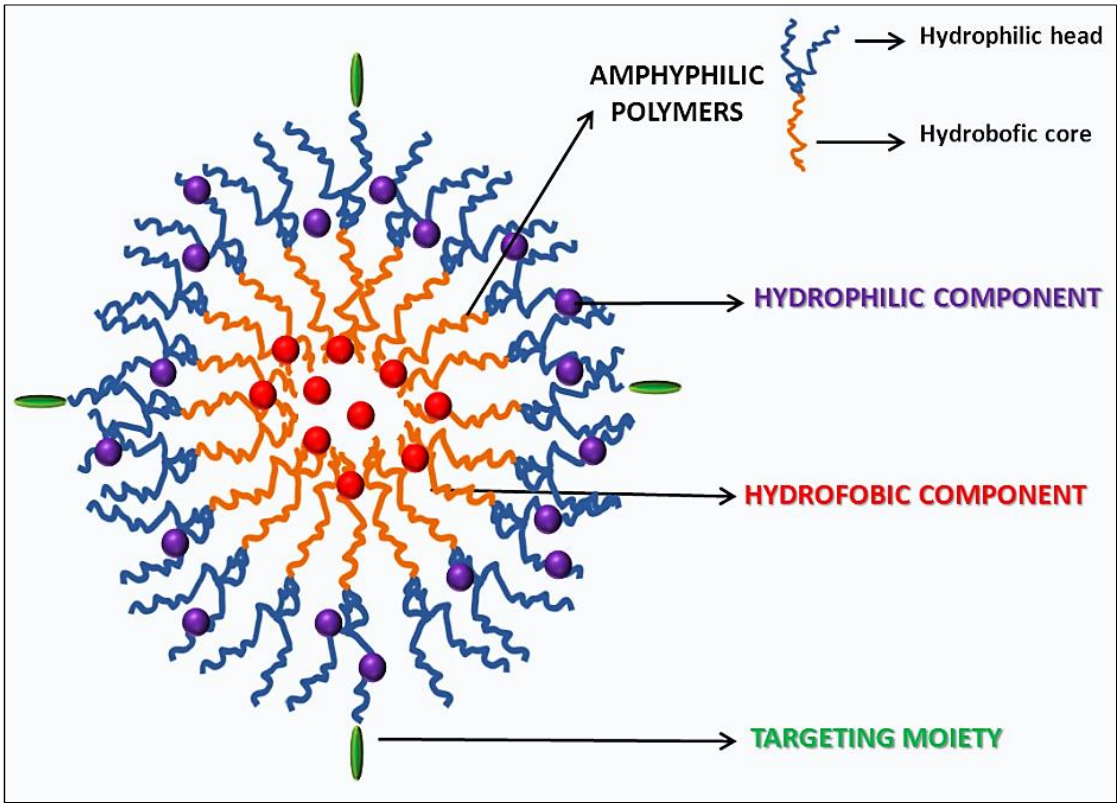


Figure 6. 1. Schematic representation of an amphiphilic polymer based multifunctional nanoparticle for gene and drug delivery combination.

References

1. Miller KD, Siegel RL, Lin CC, Mariotto AB, Kramer JL, Rowland JH, et al. Cancer treatment and survivorship statistics, 2016. CA: a cancer journal for clinicians. 2016;66(4):271-89.

2. Siegel RL, Miller KD, Jemal A. Cancer Statistics, 2017. CA: a cancer journal for clinicians. 2017;67(1):7-30.

3. Housman G, Byler S, Heerboth S, Lapinska K, Longacre M, Snyder N, et al. Drug resistance in cancer: an overview. Cancers. 2014;6(3):1769-92.

4. Gener P, Rafael DF, Fernandez Y, Ortega JS, Arango D, Abasolo I, et al. Cancer stem cells and personalized cancer nanomedicine. Nanomedicine. 2016;11(3):307-20.

5. Sehl ME, Shimada M, Landeros A, Lange K, Wicha MS. Modeling of Cancer Stem Cell State Transitions Predicts Therapeutic Response. PLoS One. 2015;10(9):e0135797.

6. Byler S, Goldgar S, Heerboth S, Leary M, Housman G, Moulton K, et al. Genetic and epigenetic aspects of breast cancer progression and therapy. Anticancer research. 2014;34(3):1071-7.

7. Tabchy A, Eltonsy N, Housman DE, Mills GB. Systematic identification of combinatorial drivers and targets in cancer cell lines. PloS one. 2013;8(4):e60339.

8. Tachtsidis A, McInnes LM, Jacobsen N, Thompson EW, Saunders CM. Minimal residual disease in breast cancer: an overview of circulating and disseminated tumour cells. Clinical & experimental metastasis. 2016;33(6):521-50.

9. Tufts Center for the Study of Drug Development. Personalized Medicine Gains Traction but Still Faces Multiple Challenges. www.csdd.tufts.edu; 2015.

10. Zeisberg M, Neilson EG. Biomarkers for epithelial-mesenchymal transitions. The Journal of clinical investigation. 2009;119(6):1429-37.

11. Makridakis M, Vlahou A. Secretome proteomics for discovery of cancer biomarkers. Journal of proteomics. 2010;73(12):2291-305.

12. Makridakis M, Roubelakis MG, Vlahou A. Stem cells: insights into the secretome. Biochimica et biophysica acta. 2013;1834(11):2380-4.

13. Rafael D, Doktorovova S, Florindo HF, Gener P, Abasolo I, Schwartz S, Jr., et al. EMT blockage strategies: Targeting Akt dependent mechanisms for breast cancer metastatic behaviour modulation. Current gene therapy. 2015;15(3):300-12.

14. Xia P, Xu XY. PI3K/Akt/mTOR signaling pathway in cancer stem cells: from basic research to clinical application. Am J Cancer Res. 2015;5(5):1602-9.

15. Yap TA, Walton MI, Hunter LJ, Valenti M, de Haven Brandon A, Eve PD, et al. Preclinical pharmacology, antitumor activity, and development of pharmacodynamic

markers for the novel, potent AKT inhibitor CCT128930. Molecular cancer therapeutics. 2011;10(2):360-71.

16. Caster JM, Patel AN, Zhang T, Wang A. Investigational nanomedicines in 2016: a review of nanotherapeutics currently undergoing clinical trials. Wiley interdisciplinary reviews Nanomedicine and nanobiotechnology. 2017;9(1).

17. Jain V, Jain S, Mahajan SC. Nanomedicines based drug delivery systems for anti-cancer targeting and treatment. Current drug delivery. 2015;12(2):177-91.

18. Ulasov AV, Khramtsov YV, Trusov GA, Rosenkranz AA, Sverdlov ED, Sobolev AS. Properties of PEI-based polyplex nanoparticles that correlate with their transfection efficacy. Mol Ther. 2011;19(1):103-12.

19. Alakhova DY, Rapoport NY, Batrakova EV, Timoshin AA, Li S, Nicholls D, et al. Differential metabolic responses to pluronic in MDR and non-MDR cells: a novel pathway for chemosensitization of drug resistant cancers. J Control Release. 2010;142(1):89-100.

20. Biswas S, Kumari P, Lakhani PM, Ghosh B. Recent advances in polymeric micelles for anti-cancer drug delivery. European journal of pharmaceutical sciences : official journal of the European Federation for Pharmaceutical Sciences. 2016;83:184-202.